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UNIVERSITY OF ALBERTA

INTERACTIONS OF GLIOCLADIUM ROSEUM AND MYROTHECIUM VERRUCARIA WITH ALTERNARIA BRASSICAE

BY

LISA ANNE BOYKO ©

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

PLANT PATHOLOGY

DEPARTMENT OF PLANT SCIENCE

EDMONTON, ALBERTA

SPRING 1995

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"All things are connected....whatever befalls the Earth befalls the sons of the Earth....This we know - the Earth does not belong to man, man belongs to the Earth....Man did not weave the web of life; he is merely a strand in it. Whatever he does to the web, he does to himself."

-Chief Seattle, Dwamish Tribe, 1854

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DATE Dec. 20, 1994

To my parents Albert and Olympia J. Boyko and my brother Adrian M. Boyko
whose love and support have made my dream a reality.

ABSTRACT

Alternaria blackspot of oleiferous crucifers occurs worldwide and is caused by several species of *Alternaria*. In Canada, this disease is usually caused by *A. brassicae* (Berk.) Sacc. and infection may result in significant economic loss to the producer. Control is usually obtained through chemical and cultural practices. There is no effective practical means of biocontrol of this disease, however certain antagonistic organisms or their byproducts are potentially promising.

Preliminary screening tests using soil fungi indicated that two isolates of *Gliocladium roseum* Bainier and one isolate of *Myrothecium verrucaria* (Alb. & Schw.) Ditmar showed antagonistic behavior towards *A. brassicae* via coiling around the conidia of *A. brassicae* and in the case of *M. verrucaria*, inhibition of germination of conidia and growth of *A. brassicae*. Further studies demonstrated that this inhibition was dependant upon the concentration of metabolites produced by and/or carried on the conidia of *M. verrucaria*.

In colony interaction studies, *A. brassicae* colonies were usually grown over by *G. roseum* and *M. verrucaria* colonies and in the case of *M. verrucaria*-*A. brassicae* co-cultures, *A. brassicae* colony growth was usually inhibited. Parasitism of *A. brassicae* by *G. roseum* and *M. verrucaria* differed on media of differing nutrient value and the severity of parasitism and the percentage of conidia of *A. brassicae* parasitized were greatest on high nutrient media. The results of fluorescence microscopy were inconclusive with respect to the viability of parasitized *A. brassicae* conidia.

Light microscopy as well as scanning and transmission electron microscopy clearly demonstrated that *G. roseum* and *M. verrucaria* are mycoparasites of *A. brassicae*. Penetration of conidia of *A. brassicae* occurs via enzymatic or enzymatic and mechanical means for *G. roseum* and *M. verrucaria* respectively.

Experiments using excised and *in situ* leaves of Legend (*Brassica napus* L.) and Reward (*Brassica rapa* L.) indicated that suspensions of washed conidia of *G. roseum* and *M. verrucaria* had no potential for biocontrol of *A. brassicae*. *Alternaria brassicae* combined with the unwashed conidia of *M. verrucaria*, delayed disease development and symptoms were less severe. The suspension of crude metabolites of *M. verrucaria* was not effective in controlling

disease caused by *A. brassicae*. Occasionally, *M. verrucaria* incited disease on the leaves of Legend and Reward.

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CHAPTER ONE: LITERATURE REVIEW

Introduction

Canola and mustard belong to the family Brassicaceae, which was formerly known as the Cruciferae. This original Latin name is still in common use today and was given to this family because the flowers are composed of four petals arranged in the form of a cross. Another family characteristic is the appearance of the 6 stamens, 4 of which are long and 2 of which are short. The fruits of the Brassicaceae are commonly referred to as pods but the proper name for these structures is siliques or silicles, which are unique to this family (Budd, 1987; Stern, 1982).

Several species of the genus *Brassica* are considered to be rapeseed. *Brassica napus* L. (rape), *B. rapa* L. (turnip rape) and *B. juncea* L. (leaf mustard) are but 3 of these species, each of which has several common names. *Brassica napus* is commonly referred to as rapeseed, oil rape, oilseed rape, swede rape and Argentine rape. Rapeseed, oil turnip, and Polish rape all refer to *B. rapa*, while *B. juncea* is commonly referred to as brown mustard, Oriental mustard or Indian mustard. In Canada and Europe, *B. napus* and *B. rapa* seeds are regarded as rapeseed (Shahidi, 1990).

Historically, rapeseed, such as the varieties grown in Asia, contain 22-60% erucic acid in their oils. These oils are known as high erucic acid rapeseed oil. The presence of such high quantities of erucic acid in the oil compromises the nutritional value of oil. The uses of meal produced by these rapeseed varieties is limited due to the presence of goiterogenic glucosinolates. Genetic modification of varieties of rapeseed grown in Canada resulted in production of low erucic acid varieties whose oil contains 5% or less erucic acid. The first of these was produced in 1968 and was referred to as a "single-low" or "single-zero" variety. By 1974, several low erucic acid, low glucosinolate ("double-low" or "double-zero") varieties were licensed. In 1979, in Canada, the name "canola" was adopted to refer to all "double-low" cultivars. A well established definition of canola is a rapeseed cultivar in whose oil there is less than 2% erucic acid and in whose defatted meal there is less than 30μmol/g of one or any combination of aliphatic glucosinolates (gluconapin, progoitrin, glucobrassicanapin and napoleiferin) (Shahidi, 1990).

Members of the Brassicaceae exhibit great diversity of use. Native Americans on the west coast of the United States used watercress (*Nasturtium officinale*) to treat liver ailments. Camelina (*Camelina sativa*) oil was used in soaps and as lamp oil (Stern, 1982). Many members of the Brassicaceae, such as cabbage (*B. oleracea* (*Capitata*)), brussels sprouts (*B. oleracea* (*Gemmifera*)), radish (*Raphanus sativus*), turnip (*B. rapa*) and rutabaga (*B. napus* (*Napobrassica*)) are edible and are cultivated in cooler temperate climates (Stern, 1982). Several common weeds such as Shepherd's Purse (*Capsella bursa-pastoris*), which is also edible and wild mustards (*B. kaber*) are members of this family (Stern, 1982). In Canada, commercial production of rapeseed began in 1942 with the oil being used as a lubricant for the Allied war efforts (Shahidi, 1990a). Today there is a large market for canola meal which contains several essential amino acids (Shahidi, 1990). Canadian canola meal exports in 1992/93 were 768 000 tonnes (Statistics Canada) with 66.2%, 29.8% and 4.0% exported to the USA, Asia, and Europe respectively. Canola meal is used domestically in rations for cattle, pigs and poultry (Downey and Bell, 1990). However, the main use of canola is as an edible oil (Figure 1.1). Since the production of the first low erucic acid cultivar, canola has become a crop of global importance with Canada now being the largest exporter in the world (Shahidi, 1990a) (Figure 1.2). In Canada, in 1994, 5.750 million hectares were seeded to canola with an estimated 2.000 million of those hectares in Alberta (34.8%). The estimated production of canola in Canada in 1994 was 7.400 million tonnes with 2.600 million tonnes being produced in Alberta (40.4%) (Statistics Canada).

The two major types of canola grown in Canada are *B. rapa* and *B. napus*. Europe is believed to be the center of origin for *B. napus* and for certain forms of *B. rapa* (Kolte, 1985; Vaughan, 1977). *Brassica napus* is grown mostly in Canada, Europe, Japan and Chile. The major areas of production of rapeseed and mustard in Asia are India, China and Pakistan. In North America, Canada is the major producer. The major areas of production in Europe are Poland, France, Sweden and Germany (Kolte, 1985).

Alternaria Blackspot

Alternaria blackspot is a major disease of oilseed rape and canola in Canada, England, France, Germany, Holland, India, Poland, Sri Lanka, Spain, Sweden and Trinidad (Tewari, 1993; Paul and Rawlinson, 1992; Kolte, 1985).

Worldwide, *Alternaria brassicicola* (Schwein.) Wiltshire, *A. raphani* Groves and Skolko, *A. alternata* (Fr.:Fr.) Kiessl. and *A. brassicae* (Berk.) Sacc. (Tewari, 1993; Paul and Rawlinson, 1992; Kolte, 1985; Tewari, 1985; Vaartnou and Tewari, 1972) cause Alternaria blackspot. On the Canadian prairies this disease is caused by *A. brassicae* and *A. raphani*, alone or in combination (Degenhardt et al., 1982), however *A. brassicae* is the more important of the two pathogens (Tewari, 1985).

Globally, Alternaria blackspot may be the most widespread disease of canola (Tewari, 1993, 1985) thus giving this disease worldwide economic importance (Tewari, 1993). When conditions are favorable, yield losses of 20% to 50% are common (Humpherson-Jones, 1992). Losses incurred from this disease result from reduced photosynthetic area in the leaves due to lesions, reduced photosynthetic time due to early senescence, premature ripening, a reduction in the 1000 grain weight and fruit shattering during harvest (Hardwick et al., 1991; Kolte, 1985; Louvet, 1958). The pathogen can grow through the silique wall to directly infect seed, resulting in major yield losses (Tewari, 1993). Reports of up to 36% loss in oil content of *B. rapa* seed have been noted (Hardwick et al., 1991; Ansari et al., 1988; Kadian and Saharan, 1983). The carbohydrate and protein content of *B. rapa* are also affected (Hardwick et al, 1991; Bandyopadhya et al., 1974; Degenhardt et al., 1974). Degenhardt et al. (1974) found that *A. brassicae* infection had no effect on the oil content of seed of *B. napus*. It has also been reported that there is no relationship between *A. brassicae* infection and the percent oil, percent protein, glucosinolate content or fatty acid profile of seed (Stovold et al., 1987). In 1989, in Alberta, there was an estimated loss of $23 750 000 due to *A. brassicae* infection (Tewari, 1993).

Species Description

Members of the genus *Alternaria* may be either parasites, saprophytes, or facultative parasites. *Alternaria brassicae* is a necrotrophic pathogen (Tewari, 1993). The fungi of this genus form dictyospores and are classified as belonging to the Fungi Imperfecti, order Hyphomycetes, family Dematiaceae (Rotem, 1994).

Ellis (1971) has described *A. brassicae* (Figure 1.3) on plants as having

> *"Colonies amphigenous, effuse, rather pale olive, hairy, the individual large conidia plainly seen under a binocular dissecting microscope. Mycelium immersed; hyphae branched, septate, hyaline, smooth, 4-8μ thick. Conidiophores arising in groups of 2-10 or more from the hyphae, emerging through stomata, usually simple, erect or ascending, straight or flexuous, frequently geniculate, more or less cylindrical but often slightly swollen at the base, septate, mid-pale greyish olive, smooth, up to 170μ long, 6-11μ thick, bearing one to several small but distinct conidial scars. Conidia solitary or occasionally in chains of up to 4, acropleurogenous arising through small pores in the conidiophore wall, straight or slightly curved, obclavate, rostrate, with 6-19 (usually 11-15) transverse and 0-8 longitudinal or oblique septa, pale or very olive or greyish olive, smooth or, infrequently, very inconspicuously warted, 75-350μ long and usually 20-30μ (sometimes up to 40μ) thick in the broadest part, the beak about 1/3 to 1/2 the length of the conidium and 5-9μ thick."*

Host Range and Symptoms

Many pathogens in this genus are of worldwide distribution while others are restricted to specific areas. *Alternaria* species occur in a wide range of environments and on a wider range of agricultural commodities. They infect a multitude of plant species and cause economically important diseases on cotton, several vegetables, linseed (Chelkowski, 1992) and several Brassicas. These diseases are difficult to control because of the omnipresence of these fungi, their short life cycle and their effective methods of dissemination (Chelkowski, 1992). *Alternaria brassicae* has a wide host range including canola, rapeseed and mustards grown around the world as well as some non-cruciferous species (Hardwick et al., 1991). Saharan et al. (1982) reported *A. brassicae* to be pathogenic on Krishaneel (*Anagallis arvensis* L.) and Hirankhuri (*Convolvulus arvensis* L.) in India. On the Canadian prairies, many common cruciferous weeds as well as cultivated brassicas act as hosts. All rapeseed cultivars are susceptible, however the Argentine cultivars which have a thick waxy layer on the leaf surface are not as susceptible as the Polish cultivars which have a less developed wax layer on the leaf surface. Within the oleiferous brassicas, no high degrees of resistance have been found (Tewari, 1993), while high degrees of resistance have been located in some wild and weedy crucifers (Tewari, 1993; Tewari and Conn, 1992; Conn et al., 1988).

Characteristic symptoms of this disease form on the leaves, stems and fruits of plants (Tewari, 1985; Kolte, 1985). The lower leaves are the first to show symptoms, which initially appear as black points that later develop into round, then concentric dry zonate spots. After infection occurs, lower leaves defoliate and disease symptoms move up the plant and are seen as spots on the middle and upper leaves. Symptoms on upper leaves usually remain small but do not always exhibit a concentric pattern. As the plants mature, spots may be seen on the stems and siliques (Kolte, 1985). In countries with temperate climates, seedling infection may occur and is manifested by the appearance of Alternaria blackspot on the cotyledons and hypocotyls (Kolte, 1985; Petrie, 1975). However, infection caused by seed-borne inoculum rarely reduces the plant stand (Humpherson-Jones, 1992: Petrie, 1974; Richardson, 1970).

Infection of plants by *A. brassicae* occurs primarily from wind-borne spores (Kolte, 1985) as well as from seed borne inoculum (Kolte, 1985; Humpherson and Maude, 1983). In the presence of moisture, germ tubes may arise from any of the cells of the spore, and in some cases all cells of the spore will germinate. Penetration of the leaf occurs through penetration of the stomata and direct penetration of the leaf, with the resulting spots developing three to four days after inoculation (Tewari, 1986; Kolte, 1985 and Tsuneda and Skoropad, 1976). Conidia formed in these spots are disseminated and cause secondary infection (Kolte, 1985).

Disease development is favored by moist, warm weather or by periods of rain and sun (Kolte, 1985; Vasudeva, 1958). Alternaria blackspot infection is usually most severe when rainy weather with high winds and over 80% humidity occurs during crop development, especially when the siliques are forming (Kolte, 1985; Chahal and Kang, 1979; Louvet, 1958). These environmental conditions facilitate dispersal and germination of conidia and subsequent infection of leaves and siliques (Kolte, 1985). There is a direct correlation between rainfall during flowering and infection rate of siliques (Kolte, 1985; Stankova, 1975). Other factors inherent in disease occurrence are temperature and age of inoculum. *Alternaria brassicae* is favoured by lower temperatures such as those encountered on the Canadian prairies (Humpherson-Jones, 1992; Kolte, 1985; Degenhardt et al., 1982). Older conidia tend to have lower germination rates than younger conidia, therefore age of the *A. brassicae* conidia is an important factor in infection (Kolte, 1985; Mukadam and Deshmukh, 1977).

Alternaria brassicae may survive on infected plant debris in the soil or on infected weeds. On the Canadian prairies overwintering of the fungus on infected canola stubble is of great importance (Tewari, 1985). Survival on seed is also common in countries with cooler climates (Kolte, 1985; Humpherson and Maude, 1983; Petrie, 1975; McDonald, 1959; Groves and Skolko, 1944). Overwintering structures known as chlamydospores (Kolte, 1985; Tsuneda and Skoropad, 1977a; Vaartnou and Tewari, 1972a; Atkinson, 1953) and microsclerotia (Kolte, 1985, Tsuneda and Skoropad, 1977a) may also be formed by *A. brassicae.*

Control of Alternaria brassicae

Control of *A. brassicae* in the field may be attempted through host resistance, chemical control, cultural control and biological control. With respect to host resistance, *B. napus* and *B. juncea*, which are digenomic species, are less susceptible than *B. rapa,* which is a monogenomic species (Kolte, 1985; Singh and Bhowmik, 1981; Bhander and Maini, 1965; Vasuveda, 1958). *Brassica napus* plants are less susceptible to infection by *A. brassicae* than *B. rapa* because of a thick layer of epicuticular wax on the leaf surface which results in a water repellent surface which in turn reduces the retention of conidia on the leaf (Conn and Tewari, 1989; Kolte, 1985; Skoropad and Tewari, 1977; Tewari and Skoropad, 1976). This wax layer is much less developed on *B. rapa* plants, hence their higher susceptibility. *Sinapis alba*, another member of the Brassicaceae, is resistant to *A. brassicae* (Kolte, 1985; Röjez, 1976; Bhander and Maini, 1965).

In the field, Alternaria blackspot is usually controlled with fungicide sprays. Dithiocarbamates, such as mancozeb or zineb used at a concentration of 0.2% exhibit effective control of Alternaria blackspot (Humpherson-Jones, 1992; Kolte, 1985; Prasard et al., 1970). The main drawback of the use of these fungicides is that they require early and frequent application (Humpherson-Jones, 1992). Of the newer fungicides used, iprodione (Rovral ®) which has been used as a seed treatment to control seedling infection of *B. oleracea* (Humpherson-Jones, et al., 1979), gives good control of *A. brassicae* in oilseed rape and the effects of this fungicide are persistent (Humpherson-Jones, 1992). Copper fungicides such as copper oxychloride and Bordeaux mixture have demonstrated effective reduction in severity of Alternaria blackspot (Chahal and Sekhon, 1981; Vasudeva, 1958). Husain and Thakur (1962) demonstrated that griseofulvin used at 50 to 500 ppm controlled disease with a single spray. Mycostatin, polyoxins and antibiotics produced by *Pseudomonas fluorescens* are also toxic or inhibitory to *A. brassicae*. (Hardwick et al., 1991; Dahiya et al., 1988; Singh and Rai, 1982; Tewari and Skoropad, 1979). Polyoxins B and D have both prophylactic and therapeutic actions against *A. brassicae* (Tewari and Skoropad, 1979) and effective control of *Alternaria* was achieved when polyoxins B and D were sprayed onto plants (Hardwick et al., 1991; Tewari and Skoropad, 1979 and Husain and Thakur, 1962). Tewari and Skoropad (1979) reported that treatment of *A. brassicae* conidia with polyoxins resulted in reduced mycelial growth and abnormal spore germination (Hardwick et al., 1991).

Cultural methods used to control *A. brassicae* in the field are crop rotation to decrease the possibility of pathogen buildup and carry over of the pathogen from one year to the next, removal of rapeseed debris, deep-ploughing of debris and removal of alternate hosts of *A. brassicae,* such as cruciferous weeds (Hardwick et al., 1991; Tewari, 1985). Early swathing of severely infected crops helps reduce losses (Tewari, 1985). These cultural methods are commonly used in Alberta. The use of clean seed and timing of planting to escape disease may also be used (Kolte, 1985).

Studies on biological control of several species of *Alternaria* through the use of antagonistic microorganisms have been ongoing for several decades. Phyllosphere residents on healthy leaves may interfere with the infection process as carried out by several species of *Alternaria*. Pace and Campbell (1974) reported that *Aureobasidium pullulans* (de Bary) G. Arnaud and *Epicoccum nigrum* Link ex Fr. parasitize *A. brassicicola* (Hardwick et al., 1991). Vannacci and Harman (1987) suggested that mycoparasitism may have an important role in the control of *Alternaria* spp. by antagonistic fungi. *Gonatobotrys simplex* Corda attacks several species of *Alternaria* in nature and has been associated with *A. tenuissima* (Kunze:Fr.) Wiltshire, *A. cucumerina* (Ellis & Everh.) J. A. Elliot, *and A. solani* Sorauer. Studies with *G. simplex* and *A. alternata* (Fr.:Fr.) Keissl. (syn. *Alternaria tenuis*) showed that there is cytoplasmic continuity between the host and parasite via membrane-lined plasmodesmata (Kwasna, 1992). Parasitic contact between *G. simplex* and *A. alternata* is established through short-branched contact cells. Contact in this system occurs in two ways. Firstly, the host and parasite may both produce short hyphal branches and make contact or the host may grow and contact *G. simplex* spores which have already germinated. Secondly, *G. simplex* may produce short branches and contact *A. alternata* in that manner (Hoch, 1977).

Alternaria brassicae and *A. raphani* are parasitized by *Nectria inventa* Pethybr. (Vannacci and Harman, 1987; Tsuneda and Skoropad, 1980; Tsuneda and Skoropad, 1977). The host parasite relationship between *N. inventa* and *A. brassicae* results in ultrastructural changes in the host cells and eventually the degeneration of these cells. *Nectria inventa* prefers to attach to the septal area or the basal portion of the germ tube. When penetration occurs at the septa, cell separation results. Within this host-parasite system, conidia are penetrated more frequently than hyphae. However, contact parasitism through the formation of an appressorium-like body also occurs (Tsuneda and Skoropad, 1977). Contact parasitism is a relationship in which the parasite forms specialized branches which establish contact with the host. The host may be completely or partially surrounded by these branches or a short branch of the host may touch tip-to-tip with the parasite branches (Manocha, 1991; Barnett and Binder, 1973).

Alternaria brassicicola conidia and hyphae are penetrated by *T. harzianum*, *Penicillium citrinum* Thom and *P. oxalicum* Curie and Thom (Wu and Lu, 1984). Fungi which coil around *A. brassicicola* include *T. harzianum, P. corylophilum* Dierckx, *P. citrinum* , and *P. oxalicum* (Wu and Lu, 1984). *Gliocladium virens, T. harzianum*, and *P. corylophilum* induced plasmolysis or granulation of conidia or hyphae of *A. brassicicola* (Wu and Lu, 1984). Mycelial growth and spore germination of *A. brassicicola* was inhibited by *A. pullulans* and *Epicoccum purparascens* Ehrenb. (Wu and Lu, 1984; Pace and Campbell, 1974).

Trichoderma hyphae coil around and grow parallel to hyphae of *A. raphani*, as do *Fusarium* species in association with *A. brassicicola* (Vannacci and Harman, 1987). *Chaetomium globosum* Kunze:Fr. shows coiling on conidia and hyphae of *A. brassicicola* and *A. raphani* and appressoria-like structures are formed. *Chaetomium globosum* induced production of reaction zones in *A. brassicicola* hyphae (Vannacci and Harman, 1987).

Mycoparasitism

Presumptive parasites which coil around hyphae of other fungi or overgrow other colonies on agar may be included when the term mycoparasite is used in the broadest sense. Antagonism by penetration of host hyphae, antibiotics, wall lytic enzymes and toxic radicals are often components of mycoparasitism. Elements which are of importance in mycoparasitism are the initial growth of the antagonist and recognition of the pathogen by the antagonist, as well as the production of cell wall degrading enzymes (Renwick and Poole, 1989). Two of the earliest reports of mycoparasitism were made by de Bary in 1865 and 1870 when he described and named *Piptocephalis freseniana* de Bary and *Cicinnobolus cesatii* de Bary and reported their mycoparasitic abilities (Barnett, 1964).

Some mycoparasites have been found to be responsible for natural suppressiveness of soils to plant pathogens while others can be applied inundatively to seeds or soil to achieve control in experimental conditions (Mulligan and Deacon, 1992). Most efficient antagonists are good competitive saprophytes (Baker, 1986).

Biotrophic and Necrotrophic Mycoparasites

Two classes of parasites exist; (1) biotrophic parasites, which may or may not be able to grow on artificial media, but obtain nutrients from a living host and (2) necrotrophic parasites which use nutrients from host cells which they have killed shortly before or after invasion. They tend to be more aggressive than biotrophic mycoparasites and have a broad host range. Their mode of parasitism is relatively unspecialized, they do not produce special absorptive structures (branches, cells or haustoria) as do the biotrophic mycoparasites. The ability of necrotrophic mycoparasites to produce antibiotics, toxins, or hydrolytic enzymes in quantities sufficient to kill the host is considered to be the basis of their antagonistic activity (Manocha, 1991). The host range of the biotrophic mycoparasites is usually more restricted than that of the necrotrophs. As well, the biotrophs produce specialized structures through which they obtain nutrients from their host (Manocha, 1991). Fungi which produce antibiotics capable of killing organisms at a distance, by diffusing through media are not included in this category (Manocha, 1991; Barnett, 1964).

Necrotrophic Mycoparasites

Many necrotrophic parasites, such as *Trichoderma lignorum* Tode (syn. Trichoderma viride Pers.:Fr.), *Papulospora stoveri* Warren, *R. solani* and *G. roseum* have similar modes of parasitism. Partial or complete coiling of the parasite hyphae around the host usually occurs after initial contact between the host and mycoparasite (Barnett, 1964).

Pythium nunn Lifshitz, Stanghellini and Baker is an example of a necrotrophic mycoparasite which exhibits a limited host range and differential modes of attack among suscepts. It produces thin, slender side branches which, during initial parasitism, grow around the surface of host hyphae. One of two types of parasitism occurs next: 1) the quick reaction or 2) the slow reaction. The quick reaction occurs when *P. nunn* coils rapidly and intensely around *P. ultimum* Trow and *P. vexans* de Bary, with the hyphae usually completely obscuring the host. Host cytoplasm then disappears over a few hours and the hyphae often burst. The slow reaction occurs with hosts such as *P. aphanidermatum* (Edson) Fitzp., *Phytophthora parasitica* Dastur, *P. cinnamomi* Rands and *R. solani* when the host is slowly and only slightly encompassed. Globular appressoria and sometimes infection pegs form. While host cytoplasm disappears, there is no hyphal bursting or massive lysis. This suggests that penetration of the host is more difficult in the slow reaction (Lifshitz et al., 1984). The basis of the two different types of reactions may lie in differences in host resistance and success of attempted parasitism.

Biotrophic Mycoparasites

Some biotrophic parasites, such as *Calcarisporium parasiticum* Barnett and *G. simplex* are deficient for a growth factor and must absorb this factor from a living host or an artificial medium to which the growth factor has been added. If this growth factor is lacking in a fungus it will be immune, however the presence of the growth factor itself does not necessarily result in susceptibility. The ability of the mycoparasite to absorb the nutrient from the living host results in the success or failure of parasitism (Barnett, 1964).

Mycoparasitic Mucorales, which often parasitize other Mucorales, are known to produce haustoria which penetrate host hyphae. When species of *Piptocephalis* contact the host, appressoria are formed, followed by penetration via an infection peg. This infection peg may remain unbranched or form a haustorium. Several of the Mucorales are parasitized by species of *Piptocephalis*. Some ascomycetes and imperfect fungi are also parasitized by species of *P. xenophila* Dobbs & English. *Piptocephalis* requires a living host to make continuous growth. This is an example of the most complex and delicately balanced host-parasite complex, where the parasite absorbs nutrients from the host with minimal injury to the host (Barnett, 1964).

Biological Control of Plant Pathogens

The idea of biological control of plant pathogens was developed by Weindling in 1932. After observing that *T. lignorum* parasitized several soilborne fungi in culture, Weindling suggested that certain pathogenic fungi could be controlled by soil augmentation with *T. lignorum* (Adams, 1990).

Since 1932, there have been numerous reports of mycoparasites and bacteria providing economical biological control. Pine stumps may be protected against *Heterobasidion (Fomes) annosum* (Fr.:Fr.) Bref. by application of *Peniophora gigantea* (Fr.:Fr.) Massee (Andrews, 1990). *Pythium oligandrum* Drechs. has demonstrated its biological control abilities against soilborne plant pathogens in the laboratory and in the field. When oospore preparations are applied as a seed treatment, the incidence of *P. ultimum* induced damping-off on cress and sugarbeet, *Mycocentrospora acerina* (R. Hartig) Deighton on carrot and *Phoma betae* A. B. Frank on sugar beet are reduced (Lewis et al., 1989). *Trichoderma viride*, *T. harzianum* and *T. longibrachiatum* have been demonstrated to effectively control root rot of pea caused by *Fusarium solani* and *R. solani* through seed treatment or infestation of soil (Diab et al., 1990). *Trichoderma viride* and *T. harzianum* are also effective in controlling tomato damping-off caused by *Pythium indicum* Balakrishnan (Krishnamoorthy and Bhaskaran, 1990).

Agrobacterium radiobacter (Beijerinck & Van delden) Conn strain K84 is used to control crown gall caused by *Agrobacterium tumefaciens* (Smith & Townsend) Conn (Andrews, 1990). The antibiotic agrocin 84 which is produced by *A. radiobacter* strain K84 and is taken up by *A. tumefaciens,* is the main means of control of *A. tumefaciens* (Jones and Kerr, 1989; Kerr and Tate, 1984; Kerr and Htay, 1974). The antibiotic terminates DNA synthesis in the recipient bacterium (Jones and Kerr, 1989; Kerr and Tate, 1984; Das et al., 1978; McCardell and Pootjes, 1976.). A major problem with the use of *A. radiobacter* strain K84 is that the plasmid responsible for inducing antibiotic production can be transferred to other agrobacteria. This prompted the construction of a strain of *A. radiobacter* (strain K1026) which is not capable of transferring its antibiotic inducing plasmid. Strain K1026 is also effective in controlling crown gall (Jones and Kerr, 1989). Parke et al. (1991) demonstrated that Pythium damping-off and Aphanomyces root rot of peas could be controlled in the field by using *Pseudomonas cepacia* (strain AMMD), *P. fluorescens* (strain PRA25) and *Corynebacterium* sp. (strain 5A) as a seed treatment.

Economics and Efficacy of Biocontrol

Biocontrol agents have not achieved the efficacy of other types of control measures (Baker, 1986) for three possible reasons. Firstly, routine, economic use of biological control is unattainable. Secondly, there is insufficient financial support, resulting in slow progress. Thirdly, there is a need for new and better ideas and efficient investigation of these ideas (Andrews, 1990). Factors which are inherent in economical biological control are (1) extensive epidemiological research on the disease in question, (2) a study of the ecology of both the target pathogen and the biological control agent, (3) a realistic way of approaching the study of biocontrol (Adams, 1990), (4) knowledge regarding production methods and proper formulation (Lewis et al., 1989) and (5) effective application of the biocontrol agent.

Cases where biocontrol is integrated with other measures makes biological control more cost effective (Baker, 1986). With respect to application, environmental conditions are of great concern. Conditions which enhance the activity of biological agents are desirable, but often difficult to obtain.

The two extremes of studying biocontrol are the most common ways of studying this phenomenon. On one hand, mass numbers of organisms may be tested to see what is suitable for a particular system. On the other hand, the accumulation of basic ecological knowledge (Andrews, 1990; Lewis et al., 1989) is gathered in order to select an appropriate antagonist and attempt a probable prediction of the outcome based on the characteristics of the system. These are complimentary approaches (Andrews, 1990) and if used as such would probably result in more biocontrol successes.

Drawbacks of biocontrol agents are that commercial formulation is difficult especially when consistent benefits are the goal. Disadvantages of biological control agents include the affect of environmental extremes on the agents and their limited shelf life. Special handling may be required to maintain viability, mutations may result in loss of efficacy and the agents are usually slow acting and do not eradicate the pathogen. The biological control agents may be specific to certain pathogens or diseases and may control the pathogen only on one crop. As well, there is a limited market size for biological control agents and they may also be incompatible with chemical pesticides (Lumsden and Lewis, 1989; Scher and Castagno, 1986). However, it may be possible to overcome this problem through genetic manipulation of the control agent, resulting in effective strains (Stasz, 1990). Not all characteristics required for an organism to be an effective biocontrol agent may be selected for in nature, therefore genetic improvement may be needed. Problems are inherent in genetic improvement because many of the desired characteristics may be genetically complex (Stasz, 1990).

The main advantage of biocontrol agents is that they are rarely toxic to humans (Scher and Castagno, 1986). Although there are certain risks inherent in biocontrol, it is deemed safe and worth the risk when compared with chemical control methods (Cook and Baker, 1983).

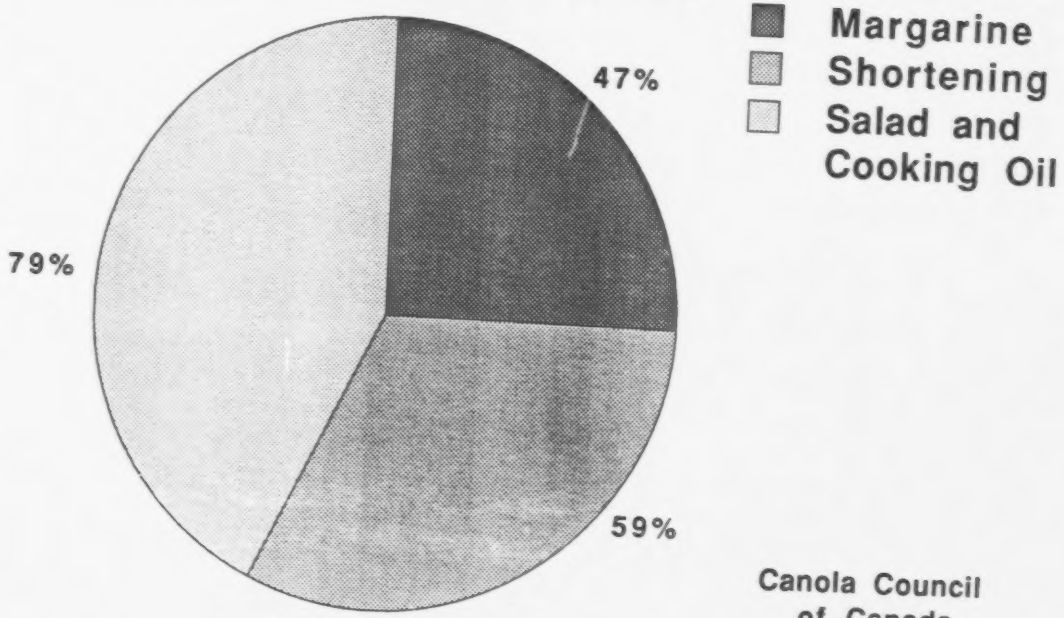
By the year 2000, the market for microbial pesticides in the Western World will grow from $33-45 million to between $6 and $8 billion (Woodhead et al., 1990).

The objectives of this thesis were to:

1) find antagonists of *A. brassicae.*

2) study the hyphal interactions of *M. verrucaria* and *G. roseum* with *A. brassicae* on *in situ* and excised leaves of *Brassica napus* cv. Legend and *Brassica rapa* cv. Reward and on media of differing nutritional value.

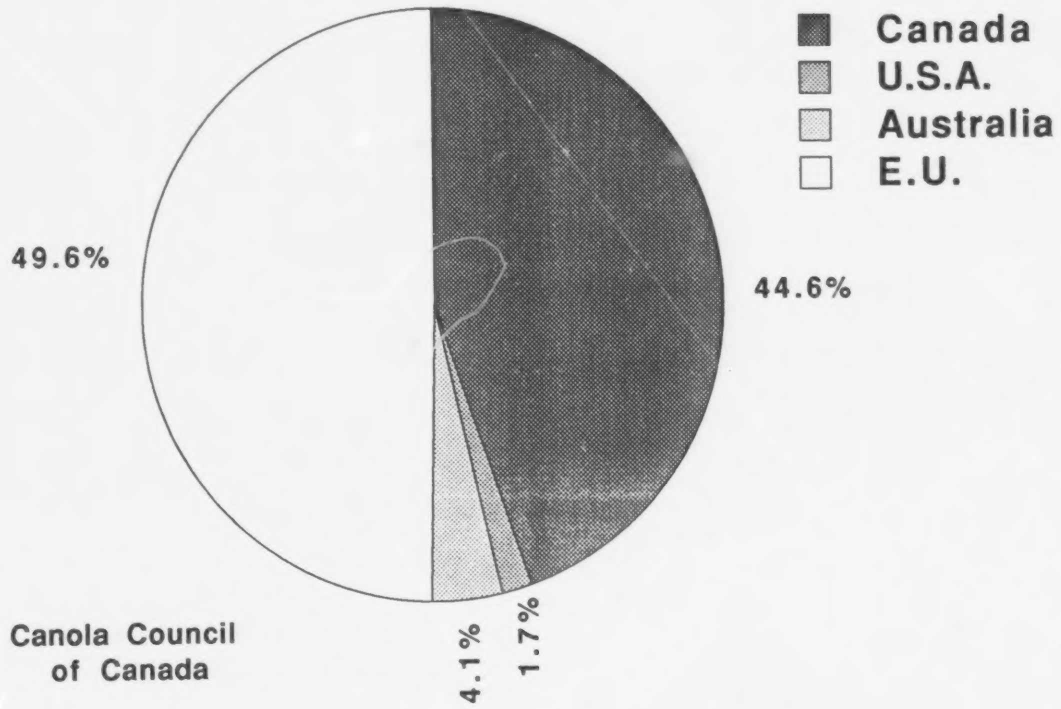
Figure 1.1. Canola Oils - Domestic Market Share Percentages (1993)



Canola Council
of Canada

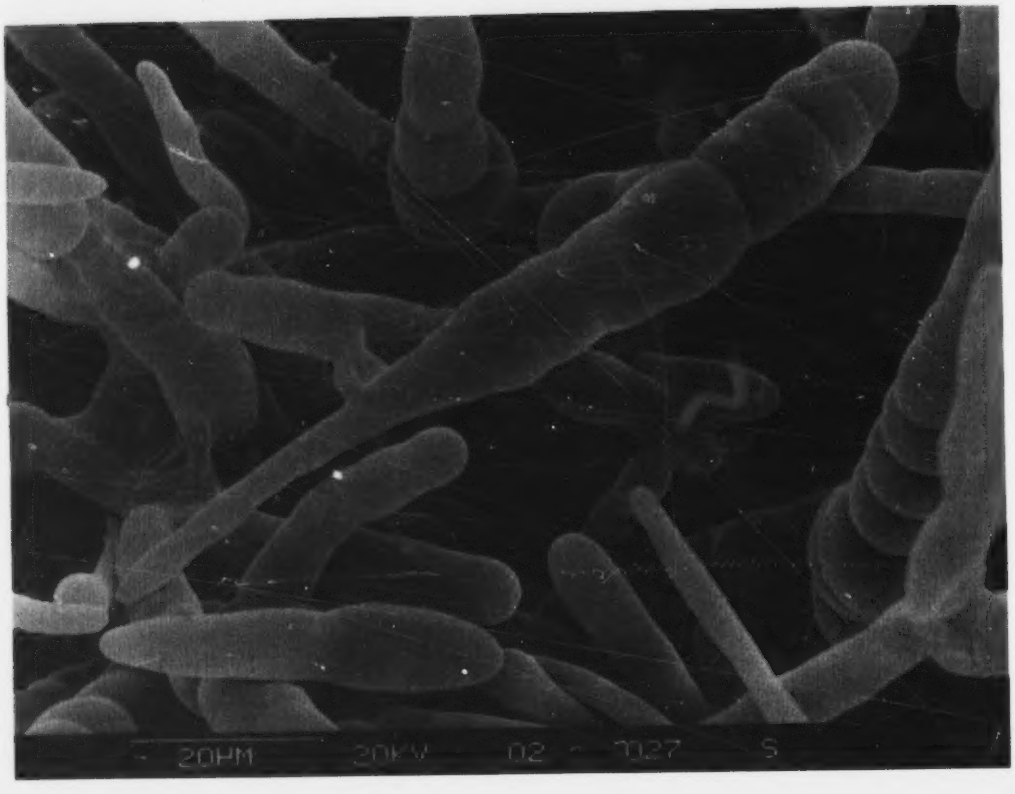
Note: Canola has over 60% of the Domestic vegetable oil market.

Figure 1.2. WORLD CANOLA PRODUCTION 93/94
12 Mn. tonnes



Canola Council
of Canada

Figure 1.3. Scanning electron micrograph of conidia of *Alternaria brassicae*.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CHAPTER TWO: BAITING EXPERIMENTS, ISOLATION OF SOIL FUNGI AND SCREENING TESTS

Introduction

Over the past few decades, there has been a dramatic increase in the importance of soil fungi as biological control agents. Several baiting techniques exist in which fruits or leaves of plants, selective media and host fungal structures may be used to isolate soil fungi such as plant pathogens or potential mycoparasites. Apple fruits and pine needles (Dhingra and Sinclair, 1985; Shepherd and Forrester, 1977; Dance et al, 1975; Campbell, 1951; Campbell, 1949) are often used to isolate *Phytophthora* de Bary species from soil. When apple fruits are used as bait, moist soil is placed in a hole in the apple which is then covered and the apple is incubated at room temperature. Isolations are then made from decaying fruits and plated, resulting in acquisition of the desired fungi. Needles of *Pinus radiata* may either be floated on water above a soil sample in an open container or may be immersed (basal portion downwards) in water above a soil sample in an open container. When the needles show symptoms of infection, they are surface disinfected and plated resulting in the growth of fungi on the medium on which they are plated. Selective media are used to obtain species of *Cylindrocladium* Morg. (Dhingra and Sinclair, 1985; Krigsvold and Griffin, 1975; Hwang and Ko; 1975) and *Aspergillus* P. Mich. Link:Fr. (Dhingra and Sinclair, 1985; Griffin and Garren, 1974; Bell and Crawford, 1967) from soil. In the case of *Cylindrocladium*, soil is wet sieved and plated on a selective medium. *Aspergillus flavus* Link:Fr. is isolated through the use of soil dilutions plated on selective media. If the causal agent of a disease is presumed to be known, infected plants, such as canola seedlings may be collected, surface disinfected and the infected regions plated on media selective for the presumptive causal agent (Calman, 1990). Burial of stacks of filter paper discs is another method used to obtain fungi from soil. Before the discs are buried they are dipped in a nutrient solution and dried. Once the appropriate incubation time has elapsed, the filter pieces are retrieved and pieces of the center disc from each stack are plated resulting in the growth of fungi from the disc. Baiting techniques in which the survival structure of the host fungus is placed on nylon and buried (Lumsden and Lewis, 1989; Kenerly et al., 1987; Ayers and Adams, 1981) are commonly used to obtain prospective biocontrol agents. Of the many fungi obtained through this technique, species of *Gliocladium* were consistently obtained and usually showed mycoparasitic potential (Kenerly et al., 1987). Lumsden et al. (1987) used a similar technique with the mycelia of *Pythium aphanidermatum* (Edson) Fitzp., which was grown on nylon and then floated on soil extract indicating that baiting techniques need not be restricted to burial of survival structures in the soil. These baiting techniques favor isolation of mycoparasites (Lumsden and Lewis, 1989). An obvious advantage to the use of baiting techniques is that at least some of the organisms isolated from fungal structures used for bait will be specific antagonists of the fungus used as bait. This may in turn result in the need for less screening to find potential antagonists. For this reason, baiting techniques involving the burial of *Alternaria brassicae* (Berk.) Sacc. conidia were chosen for the studies described in this chapter.

Materials and Methods

Baiting Experiments

Canola stem pieces and V-8 agar plates were inoculated with *A. brassicae* and kept in the dark at room temperature. In the case of canola, stem pieces (straw) were cut with a razor blade into sections 1.5 cm x 0.5 cm, placed in moist chambers (glass petri plates measuring 10 cm in diameter, containing paper towel moistened with distilled water), autoclaved and inoculated using 14-day-old *A. brassicae* conidia. Chambers and newly inoculated V-8 plates were placed in the dark at room temperature for 14 days. When the chambers began to dry, they were rehydrated with sterile distilled water. Each moist chamber contained 10 stem pieces.

After 14 days, *A. brassicae* conidia were harvested from V-8 plates by flooding the plate with sterile distilled water and gently rubbing the spores with a sterile 10 mL pipette. The suspension of conidia was then centrifuged twice in a Sorvall GLC-1 centrifuge for 4 minutes at 2000 rpm to remove any inhibitory compounds. The supernatant was poured off and the pellet of conidia was resuspended and collected in a sterile beaker. Spore concentration was then determined to be 1.7×10^6 spores/mL.

Using a sterile pasteur pipette a drop of suspension, approximately 50 µL, was placed on a piece of 44 µm nytex approximately 1 cm in diameter. Six of these nytex pieces were placed on a piece of nytex measuring 5 cm by 10 cm. Silicone seal (General Electric Supersealant-Do-It-Yourself Rubber) was spread around the 4 edges of the nytex which was then folded over and the edges pressed together to make a seal. The pockets were stored overnight at 4°C to keep the activity of *A. brassicae* to a minimum while the silicone seal set. They were then placed in baskets and buried at the University of Alberta Edmonton Field Research Center at 5 depths (surface, 2 cm, 4 cm, 6 cm, and 8 cm) at 15 cm intervals (Figure 2.1). The baskets which contained the pockets were of 5 different heights, but all with a base of 8 cm x 8 cm and were made from either nytex or vinyl coated polyester (patio #6 white). A small amount of soil was placed in the bottom of each basket. One pocket was then placed in each basket and covered with the appropriate amount of soil. Inoculated stem pieces were treated in the same manner as the nytex discs. The purpose of this experiment was to determine if different organisms at different depths were antagonistic towards *A. brassicae*.

Baiting experiments were also carried out in the greenhouse with soils collected from Alberta and Saskatchewan. The temperature of the greenhouse was 20°C daytime and 18°C nighttime. Stem pieces and nytex pieces were prepared in the same manner as for the field experiment. Pockets contained 3 stem pieces and 3 nytex pieces each. All materials were sterilized by autoclaving or by washing with 95% EtOH or household bleach. Pots (10 cm, green plastic) and tags were sterilized in a bleach solution for 30 minutes. Pockets were left overnight in a 4°C coldroom while the silicone seal set. Pots were half filled with soil, one pocket was placed in each pot and then the pots were filled with soil and tagged. Soils were collected from:

Blaine Lake	Saskatchewan
Cecil	Saskatchewan
Lashburn	Saskatchewan
Maidstone	Saskatchewan
Paynton	Saskatchewan
Kitscoty	Alberta
RR225 (Sherwood Park) (two locations)	Alberta
17 St. and Sherwood Park Freeway	Alberta

76 Ave. and 17 St.   Alberta
(Sherwood Park)

All locations had canola cropped to them during the year of collection, or the year prior to collection. All baiting pots were moistened every 3 days with approximately 100 mL of sterile distilled water.

Isolation of Soil Fungi

Upon retrieval of pockets, one nytex disc with *A. brassicae* conidia from each depth was placed in a test tube containing 5 mL sterile distilled water. The tubes were vortexed for 1 minute and 1 mL of suspension was pipetted onto each of the four plates and spread evenly over the surface. Two V-8 and 2 PDA plates were made (one of each type of plate contained Penicillin-G and Streptomycin sulphate each at a concentration of 100 mg/L). The same procedure was followed for the stem pieces. After initial plating, fungi were transferred to either PDA or V-8 plates to obtain pure cultures which were maintained on PDA slants in a 4°C cold room.

Screening Tests

Alternaria brassicae spores were harvested as previously described, while spores of the test fungi were harvested by adding approximately 1 mL of sterile distilled water to the culture and rubbing off the spores and/or mycelium with a micropipette, then retrieving 40 µL of suspension. Ten-day-old *A. brassicae* grown on V-8 and 14-day-old potential antagonist cultures grown on PDA were used.

In order to test soil fungi for antagonistic behavior towards *A. brassicae*, three types of screening techniques were carried out. In each case, sterilized cellophane strips were placed on 1% agar plates. In order to rinse off any coating which may have been present, cellophane strips were soaked in distilled water for 1 hour prior to being placed in fresh distilled water and autoclaved for 40 minutes. Two strips were placed parallel to each other on each plate and left overnight to allow the cellophane to dry before inoculation. The screening techniques were:

1) Mixtures: suspensions of *A. brassicae* mixed with other fungi
2) Side by side method: suspensions of test fungi were placed on either side of a suspension of *A. brassicae* conidia

3) Plating method: *A. brassicae* conidia were streaked across the end of a plate and the potential antagonist was placed 3 cm from *A. brassicae*

For tests (1) and (2), 40 µL droplets of suspension of each fungus were placed on the cellophane. In the case of the third test, *A. brassicae* spores were streaked onto 1% agar plates. Other fungi were transferred by means of a piece of inoculated agar, approximately 4 mm^3.

As this was a screening experiment, concentrations of potential antagonists were not determined. Concentrations of *A. brassicae* were approximately 1.5×10^5 spores/mL.

After 48 hours cellophane strips were mounted on slides, stained with lactophenol cotton blue and observed by light microscopy. Fungi which showed no antagonism towards *A. brassicae* were identified to the genus level. As many of these fungi had very fragile fruiting structures, slide cultures were made to minimize damage to the fruiting structures of the fungi.

Slide cultures were prepared in moist chambers (made as previously described) containing one sterilized glass slide each. A block of inoculated PDA was placed on a slide and a cover slip placed on top of the agar. Chambers were kept in the dark at room temperature until the fungi produced fruiting structures (10 to 21 days) at which time the cover slip was removed and placed on a drop of lactophenol cotton blue. The inoculated PDA was removed from the slide and the slide was stained with lactophenol cotton blue, resulting in two mounts of each fungus per slide. Moist chambers were rehydrated with sterile distilled water.

Maintenance of Cultures

Alternaria brassicae, *M. verrucaria* and both *G. roseum* isolates were maintained in the dark at room temperature on V-8 agar supplemented with rose bengal, potato dextrose agar and Czapek's agar respectively.

Results

Baiting Experiments and Isolation of Soil Fungi

Countless fungal colonies and several bacterial colonies were obtained as a result of the baiting experiments and isolation of organisms from the retrieved stem pieces and nytex discs. Approximately 300 fungal isolates were obtained from the baiting experiments at the University of Alberta Edmonton Field Research Center. Fourteen fungal isolates were obtained from field soil collected at 17 St. and the Sherwood Park Freeway. As many pure fungal colonies as possible were obtained from this mass of organisms and those fungi obtained from the other collected field soils and were used in the screening tests. Fungi used in the screening experiment were chosen randomly from the different depth and stem/spore combinations and from the various soils collected from the different fields. Fifty fungi were screened against *A. brassicae* and screening was halted when antagonists were found. Those fungi which were not antagonistic towards *A. brassicae* are listed in Table 2.1. Five bacteria were also screened for antagonistic behaviour towards *A. brassicae*, but no antagonism was demonstrated. These bacteria were not identified.

Screening Tests

Of all the soil fungi from the University of Alberta Edmonton Field Research Center and the collected field soils screened against *A. brassicae*, only *Gliocladium roseum* Bainier isolates 98 and 17 were found to be antagonistic towards *A. brassicae*. Isolate 98 was obtained from stem pieces buried at a depth of 6 cm at the University of Alberta Edmonton Field Research Center. Isolate 17 was obtained through the greenhouse baiting experiment from field soil located at 17St. and the Sherwood Park Freeway. *Gliocladium roseum* isolate 98 was the only fungus obtained from the stem pieces buried at that location at the University of Alberta Edmonton Field Research Center. In the case of *G. roseum* isolate 17, four other fungal isolates obtained from that pot in the greenhouse baiting experiment had colony morphology similar to *G. roseum* isolate 17 and therefore may have been the same fungus isolated repeatedly. The number designations for these isolates refer to the location from which they were isolated and are not official strain designations.

Gliocladium roseum may be identified by its conidiophores which originate from aerial hyphae and are of a penicillate fashion (Figure 2.2). The phialides are usually clumped together, but may be somewhat divergent. The conidia, which measure 5-6 x 3-4 μm, are hyaline, nonseptate and usually ovoid or kidney shaped and are usually found in clumps at the ends of the phialides. This description coincides with those provided by Barron (1983) and Domsch et al. (1980). A verticillium state is also produced by *G. roseum*

(Seifert, 1985; Barnett and Hunter, 1972)

The *Myrothecium verrucaria* (Alb. & Schw.) Ditmar culture used in this research was supplied by Dr. Tewari and was previously isolated from canola seedling roots.

Myrothecium verrucaria is easily identified by its sporodochia (Figure 2.3) which appear as wet, black spore masses with white margins. Conidiophores, which branch repeatedly, arise from a knot of basal hyphae. Phialides are borne on the ultimate branches of the conidiophores and are packed in a dense parallel layer. The one-celled conidia are hyaline and usually pointed at one end, measuring approximately 6.5-8 x 2-3.5 µm on average. A fantailed appendage is visible on the pointed end of the conidia when conidia are treated with erythrosin in 10% ammonia solution. This description is consistent with those of Cook and Baker (1983) and Tulloch (1972).

Discussion

Several different techniques are used to obtain bacterial and fungal colonies from soil. If a particular organism is desired, media selective for that organism may be used (Park et al., 1992). For this experiment, no particular organism was sought after to be tested against *A. brassicae*. The use of V8 and PDA plates proved fruitful and dilution plating was not deemed necessary, although it is commonly used when soil organisms are isolated. The antibiotics used effectively reduced or prevented the growth of bacterial colonies thereby making it easier to obtain pure fungal cultures.

The screening tests used proved to be effective methods for obtaining antagonists of *A. brassicae.* Screening tests (1) and (2) were more effective because the fungi were placed in close proximity. The effectiveness of screening test (3) was somewhat reduced in comparison to the other screening tests. A possible reason for the lack of effectiveness of test (3) is that even though the fungal colonies were intermingling there was not necessarily any antagonism demonstrated. This may be due to the distance the fungi had to grow to contact each other. The low nutrient status of this medium may have resulted in a less aggressive antagonist which did not vigorously attack its host when extensive growth was required to reach the host. Also, the age of the antagonist hyphae may have had a role in the decrease of parasitism seen in screening test (3). A discussion of these phenomena will be presented in

Chapter 3.

When the slides from the screening tests were observed, *G. roseum* was seen coiling around *A. brassicae* and appeared to have no affect upon the germination of *A. brassicae* conidia and subsequent growth of *A. brassicae*. *Myrothecium verrucaria* also coiled around *A. brassicae* and was noted to have a powerful affect upon the germination of *A. brassicae* conidia and subsequent growth of *A. brassicae*. Where *A. brassicae* conidia were associated with a high concentration of *M. verrucaria* conidia, germination of *A. brassicae* conidia and subsequent growth of *A. brassicae* was greatly reduced or completely inhibited. If few *M. verrucaria* conidia were associated with *A. brassicae* conidia, the germination of *A. brassicae* conidia and subsequent growth of *A. brassicae* was not inhibited. A discussion of this phenomenon will be presented in Chapter 3. Photographs showing the associations of *A. brassicae* with *M. verrucaria* and *G. roseum* are presented in Chapter 3.

Conclusions

The baiting, isolation and screening techniques used were effective. The antagonism of *M. verrucaria* and both *G. roseum* isolates that was demonstrated towards *A. brassicae* indicates that these fungi have potential use as biocontrol agents.

Figure 2.1. A portion of the field plot at the University of Alberta Edmonton Field Research Center showing the arrangement of a portion of the baiting baskets.

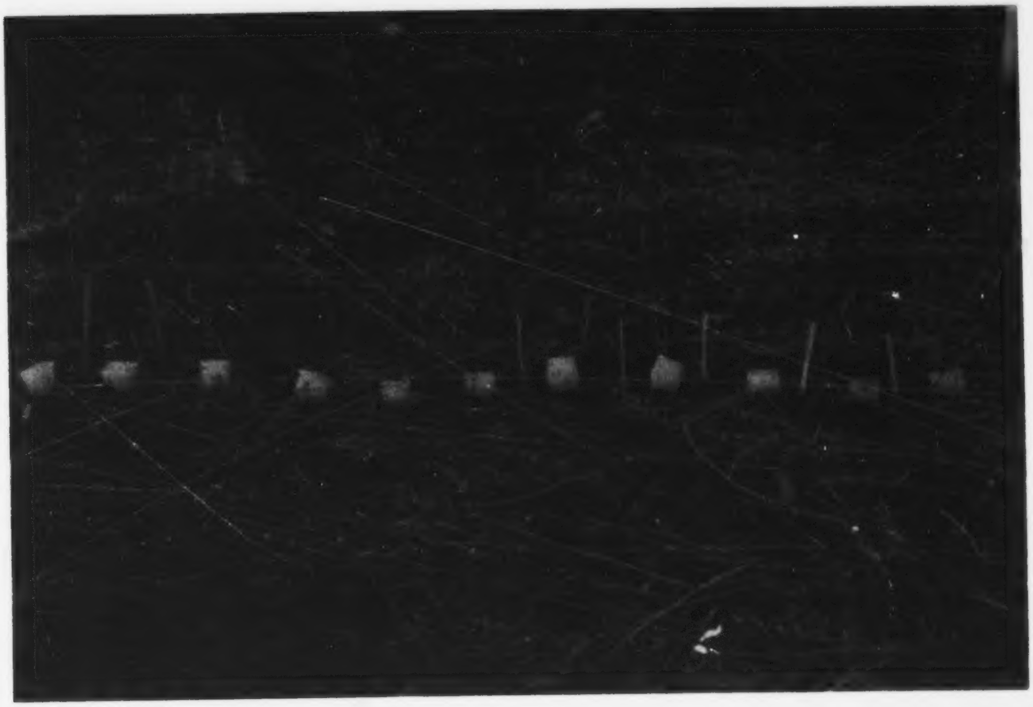
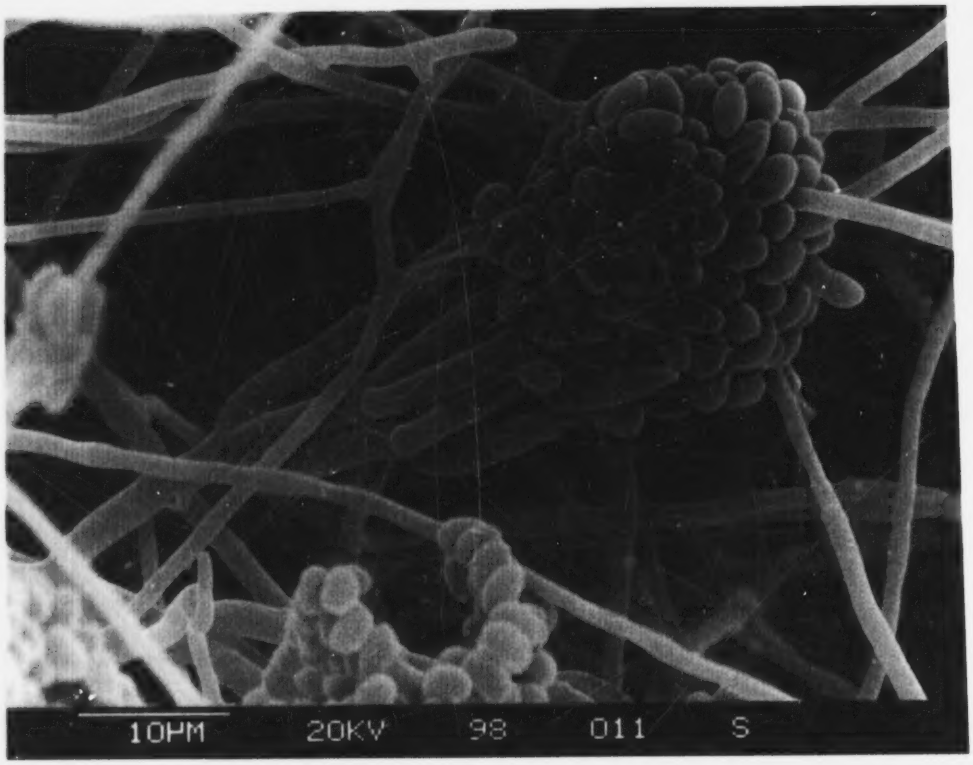


Table 2.1 Fungi Non-Antagonistic Towards *Alternaria brassicae*

Genus	Number of Isolations
Alternaria	1
Arthrobotrys	1
Aspergillus	1
Cladosporium	5
Fusarium	3
Gonatobotrys	1
Helicocephalum	1
Penicillium	13
Ulocladium	1
Mycelia sterilia	1

This list of genera is incomplete due to the fragile nature of the fruiting bodies which often broke apart when slides were made, thus usually making identification impossible. One of the fungi non-antagonistic towards *Alternaria brassicae* did not produce any fruiting bodies and is referred to as *Mycelia sterilia.*

Figure 2.2. Scanning electron micrographs showing the penicillate phialides of *Gliocladium roseum* (top) and a whorl of phialides originating from a conidiophore (bottom).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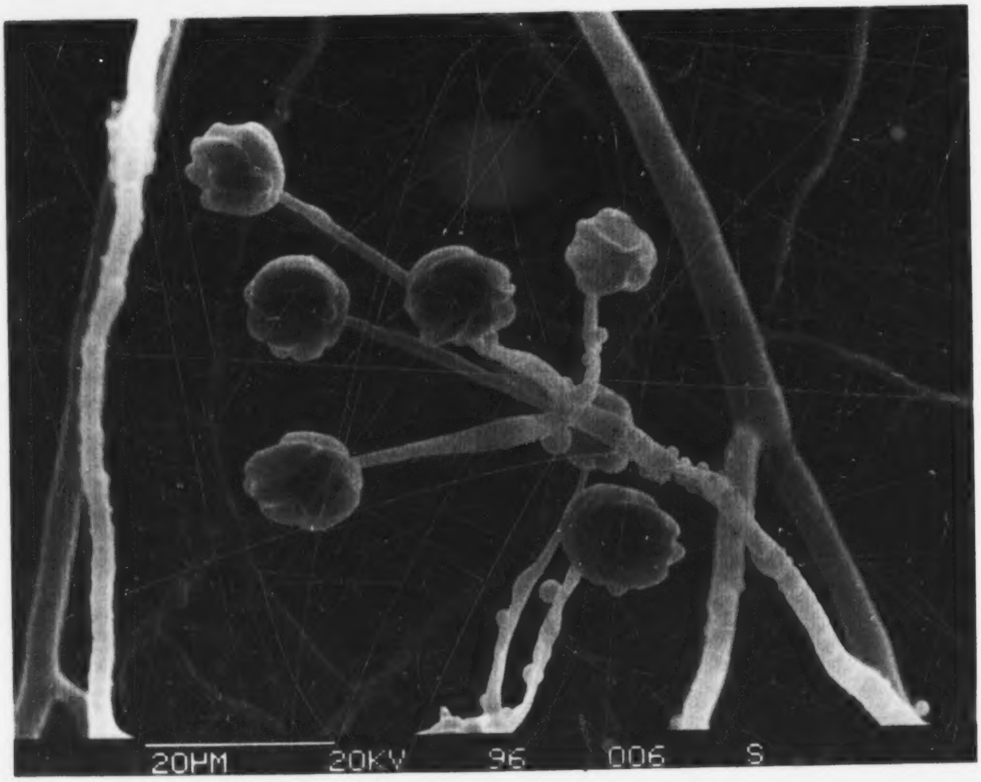
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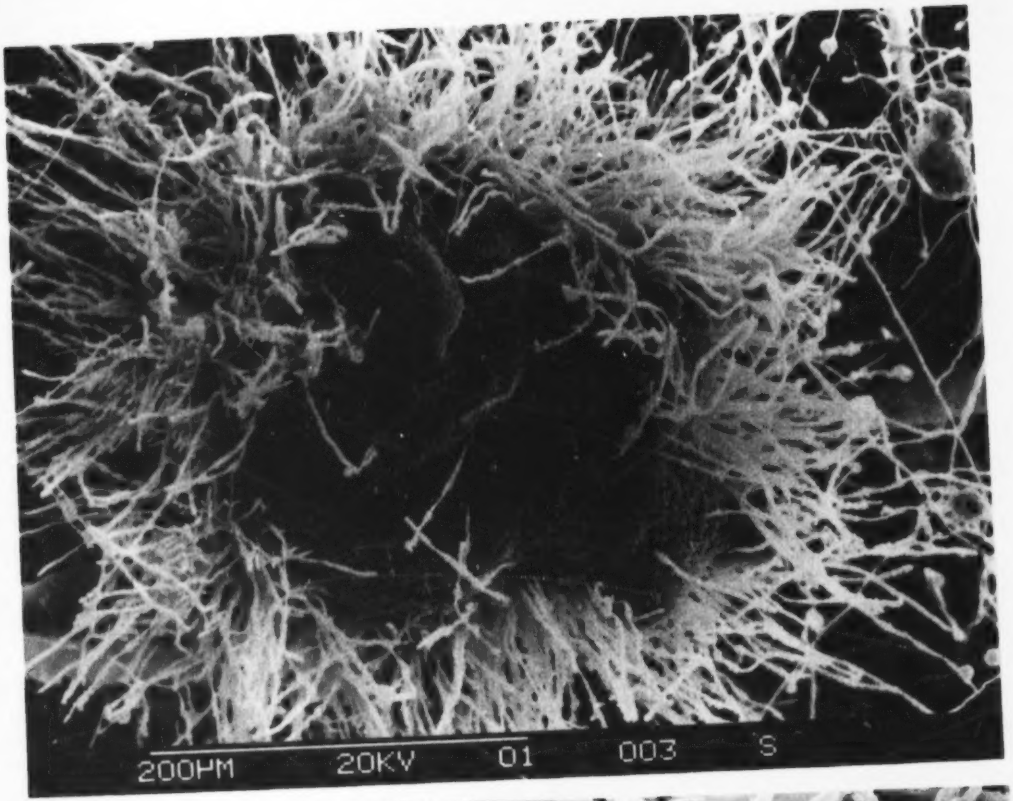
Figure 2.3. Scanning electron micrographs showing a sporodochium of *Myrothecium verrucaria* (top) and a young sporodochium of *M. verrucaria* showing conidia and phialides (bottom).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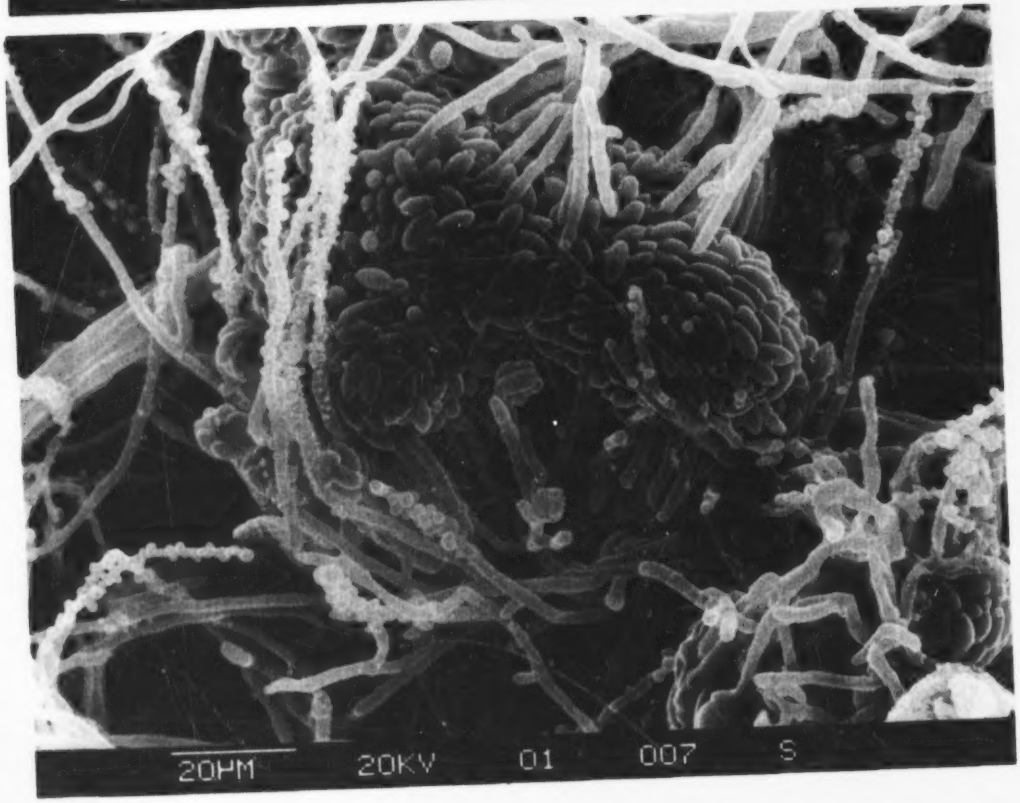
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CHAPTER THREE: EFFECTS OF MYROTHECIUM VERRUCARIA AND GLIOCLADIUM ROSEUM ON THE GROWTH OF ALTERNARIA BRASSICAE AND INTERACTIONS OF THESE FUNGI AS OBSERVED THROUGH LIGHT, FLUORESCENCE AND ELECTRON MICROSCOPY

Introduction

Fungi produce many metabolites, which are sometimes referred to as mycotoxins (Busby and Wogan, 1985; Smith and Moss, 1985). Mycotoxins are fungal metabolites which are effective against animals and humans, while phytotoxins are fungal metabolites which are effective against plants. *Myrothecium verrucaria* (Alb. & Schw.) Ditmar is known to produce secondary metabolites which act as mycotoxins and belong to the family of toxins known as the trichothecenes. In general, the trichothecenes exhibit antibacterial, antiviral and antifungal activity as well as cytostatic and cytotoxic activity. Phytotoxicity is exhibited by some trichothecenes (Cole and Cox, 1981; Desjardins, 1992), while all trichothecenes demonstrate a certain degree of animal toxicity, including insecticidal activity (Cole and Cox, 1981). This combination of mycotoxic and phytotoxic abilities makes the trichothecenes a unique group of toxins in that few toxins have this spectrum of activity. Toxic symptoms of the trichothecenes in humans and animals include severe skin irritation; hematological disorders (e.g. increase in white blood cells); vomiting and feed refusal; damage to the hematopoietic tissues in bone marrow, spleen, thymus, and lymph nodes and inhibition of the immunological response (Hesseltine, 1983). The trichothecenes are host nonspecific phytotoxins (Desjardins, 1992). Circumstantial evidence suggests that the trichothecenes play a role in plant pathogenesis. Evidence includes a correlation between virulence and the ability to produce toxins *in vitro* (Manka et al., 1985) as well as isolation of toxins from diseased plant tissues (Miller and Greenhalgh, 1988; Marasas et al., 1984). Even when treated with very low concentrations of trichothecenes (10^{-5} to 10^{-6} M) (Kuti et al., 1989; Cutler and Jarvis, 1985), a wide variety of plants show chlorosis, necrosis and other symptoms (Desjardins, 1992). Naphthazarins, a group of toxins produced by several species of *Fusarium*, have been demonstrated to cause wilting and chlorosis in treated plants (Desjardins, 1992; Nenec et al., 1978; Kern, 1972). Fusaric acid, which is produced by several *Fusarium* species, has been demonstrated to cause wilting and necrosis in plants when treated with concentrations of 10^{-3} to 10^{-4} M fusaric acid (Desjardins, 1992; Kuo and Scheffer, 1964). Kuti et al. (1989) found that roridin E, (one of the trichothecenes produced by *M. roridum*), caused lesions on leaves of Muskmelon which were similar to those caused by *M. roridum.*

Another group of fungal metabolites, known as the destruxins possess both mycotoxic and phytotoxic properties. Destruxins A and B have been demonstrated to have affects upon desert locust and tobacco budworm, respectively. Destruxin A has been demonstrated to affect fluid secretions from Malpighian tubules of the desert locust (James et al., 1993) while destruxin B affects the skeletal muscles of the tobacco budworm (Bradfisch and Harmer, 1990). Homodestruxin B and destruxin B are two phytotoxic compounds produced by *Alternaria brassicae* (Berk.) Sacc. Both compounds exhibit similar phytotoxic properties on leaves of *Brassica napus* L. However, on *A. brassicae* non-host plants, homodestruxin B caused symptoms which varied in severity. It was therefore concluded that homodestruxin B is a non-host-specific phytotoxin (Bains, et al., 1993), while destruxin B is a host-specific toxin (Shivanna and Sawhney, 1993).

In order to better understand the relationships between groups of fungi, it is essential to gain some knowledge of their interactions (Stahl and Christensen, 1992; Christensen, 1989; Boddy, 1988). Observations of interactions of colonies on different media are of interest and may provide important information on the relationships of fungi. Skidmore and Dickinson (1976) found that several different types of interactions occur between fungi growing on media in petri plates. Three such relationships are neutral intermingling of fungi, invasion of domain and deadlock. Behavior of fungi is usually affected by the nutrient level of the substrate on which they are growing (Stahl and Christensen, 1992). Changes in the antagonistic behavior due to nutrient composition are easily observed through light microscopy.

Once the antagonistic relationship has been observed one of the first questions that comes to mind is whether the host has been killed by the antagonist. An interesting and relatively simple way to determine the answer to this question is through the use of fluorescence microscopy.

Due to precision, rapidity and ease of use, fluorescence microscopy, with its potential applications of ecological, mycological and pathological studies of fungi may replace or complement vital stains to determine viability of fungal propagules (Wu and Warren, 1984). This technique can be both qualitative and quantitative. It is possible to directly compare morphology of conidia under light and fluorescence microscopes because there is usually a morphological difference between dead and viable spores. Therefore, it may be possible to use this technique to relate spore morphology to death. By quantifying the number of spores fluorescing it is also possible to determine the percentage of viable spores of a population (Wu and Warren, 1984), making fluorescence microscopy a very useful tool in the study of mycoparasitism.

Fluorescein diacetate (FDA) is commonly used as an indicator of viability (Firstencel, 1989 and Soderstrom, 1977). FDA is actually a non-fluorescent substrate, but upon penetration of cells and hydrolysis by different enzymes, fluorescein is produced. It is the fluorescein which remains in the cell causing fluorescence (Barak and Chet, 1986; Rotman and Papermaster, 1966). FDA has been used in studies of total microbial activity in the soil (Schnurer and Rosswall, 1982) and in investigations of viability of animal cell cultures (Rotman and Papermaster, 1966). The viability of plant cells, bacteria, algae, protozoa and fungi have also been studied using FDA (Soderstrom, 1977; Windholm, 1972; Medzon and Brady, 1969).

Counter stains are commonly used in fluorescence microscopy. Propidium iodide (PI), although usually used in determinations of DNA content (Eilam et al, 1992; Shapiro, 1988), has been used to assess the viability of spores of entomophagous fungi (Firstencel et al.,1990). PI passes through the membranes of dead or dying cells only, where it intercalates with the DNA and RNA thus forming a fluorescent complex (Jones and Senft, 1985).

Another intriguing question which arises during observation of antagonism through the use of light microscopy is what are the intimate details of the relationship between the host and antagonist. Through scanning electron microscopy it is usually possible to determine the details of the relationships that are occurring outside the host walls, but the question of the mode of penetration and what changes may be occurring inside the host remain unanswered unless observed through the use of light or transmission electron microscopes (Tsuneda and Skoropad, 1976) in conjunction with sectioning techniques.

Materials And Methods

Conidial Suspensions and Crude Toxin

Alternaria brassicae (Berk.) Sacc., *Gliocladium roseum* Bainier and *M. verrucaria* conidia were harvested as described for *A. brassicae* in Chapter 2. For experiments involving unwashed conidia of *M. verrucaria*, the conidia were harvested as previously described, but not centrifuged. All spore concentrations were set using a haemocytometer.

In order to obtain crude toxin (henceforth referred to as metabolites of *M. verrucaria*) from *M. verrucaria* cultures, conidia from three petri plate cultures (14-days-old) were harvested as previously described and brought up to a volume of 200 mL with sterile distilled water. This volume resulted in a spore concentration similar to that used when conidial suspensions of *A. brassicae* and *M. verrucaria* were combined. This conidial suspension was agitated and filtered through a #202 filter, Whatman #1, 2, and 3 filters and then a series of millipore filters (0.8, 0.45 and 0.22 µm). Aliquots consisting of 10 mL of metabolites of *M. verrucaria* were stored in plastic scintillation vials at -18°C. Before use, metabolites of *M. verrucaria* were filtered 3 more times through 0.22 µm millipore filters and collected in sterile flasks. To ensure that the liquid was free of conidia, 0.5 mL was plated onto each of 3 PDA plates which were observed for growth of *M. verrucaria*.

Germination Studies

Seven to ten-day-old cultures of *A. brassicae* and 14-day-old cultures of *G. roseum* (isolate 98) and *M. verrucaria* were used. Metabolites of *M. verrucaria* were also used as a treatment. *Alternaria brassicae* stock suspension was approximately 1.0×10^6 spores/mL while those of *G. roseum* and washed and unwashed conidia of *M. verrucaria* were approximately 1.0×10^7 spores/mL. The following treatments and controls were made from the stock suspensions. Three concentrations (approximately 1×10^6, 1×10^5, and 1×10^4 spores/mL) of *M. verrucaria* (washed and unwashed conidia) and *G. roseum* conidia were combined with a single concentration of *A. brassicae* conidia (approximately 1×10^5 spores/mL). Metabolites of *M. verrucaria* were serially diluted and were combined with the previously mentioned concentration of *A. brassicae* conidia. However, the starting concentration of metabolites of *M.*

verrucaria was unknown. Therefore, for consistency, the concentrations of the metabolites of *M. verrucaria* used were designated as 10^6, 10^5 and 10^4. It was believed that the concentration of the metabolites present when at the various dilutions was similar to the concentration present on unwashed conidia of *M. verrucaria* at concentrations of 10^6, 10^5 and 10^4 spores/mL. Treatments were *A. brassicae* combined with each concentration of the antagonists and with metabolites of *M. verrucaria*, while *A. brassicae* alone, antagonists alone and metabolites of *M. verrucaria* at each concentration served as controls. Due to space restrictions only one *A. brassicae*/antagonist or metabolite combination experiment could be carried out at any time. Two hundred µL of spore suspension were placed on cellophane strips on 1% water agar and incubated in the dark at 23 ± 2°C. Slides were made every 2 hours starting with time zero and ending at 8 hours, by which time *A. brassicae* had reached maximum germination. Time zero was considered to be when all plates had spore suspension applied to them. In all cases a germinated spore was considered to be one which had a distinct bulge.

Four slides were made for each treatment at each time. Slides were separated into three sections, each 1 cm^2, with 50 conidia of each fungus observed per section. Due to the consistency of data observed in the first block, the number of observations for the second block was reduced to 4 slides with only the center box on each slide being observed.

The percent of conidia germinated, percent of cells germinated and germ tube lengths were recorded for *A. brassicae*. All of the germ tubes produced by every fifth *A. brassicae* conidium were measured using an eyepiece micrometer. Percent germination of conidia was recorded for *G. roseum* and for washed and unwashed conidia of *M. verrucaria*.

Gliocladium roseum isolate 17 was not used in this study because of its low level of spore production which resulted in an inability to achieve the required stock concentration.

Colony Interactions

Colony interactions were observed on V-8, PDA, CD and 1% water agar. Four mm plugs of all fungi were used as inoculum and were cut with a cork borer. Agar plugs of 10-day-old *A. brassicae,* due to its slow growth, were plated spore side down 4 days in advance of *M. verrucaria* and *G. roseum* which were plated spore side up. The *A. brassicae* plug was placed spore side down so that *A. brassicae* did not have to grow down the plug to the media. This meant that *A. brassicae* colonies could establish themselves before antagonist colonies approached. Plugs of antagonists were placed spore side up because they grew quickly and the time required for them to grow down the plug gave *A. brassicae* colonies more time to establish growth. Fourteen-day-old antagonist cultures were used. The plugs of fungi were placed 3.0 cm apart. Controls used were single and dual inoculated cultures of the same fungus (all fungi were used as controls on all media). Four replicates of each treatment were used and the experiment was repeated once. Plates were incubated in a growth chamber at 23 ± 2°C. Interactions were assessed after 5, 10 and 15 days (Skidmore and Dickinson, 1976).

In order to view the hyphal interactions of the colonies at the light microscope level, Scotch tape was pressed onto the colonies where they met. The tape was mounted on a glass slide and stained with lactophenol cotton blue. One slide was made for each plate fifteen days after inoculation with the antagonist.

Parasitism Experiments

The concentrations of spore suspensions used for this experiment were approximately $2.5X10^5$ spores/mL for *A. brassicae* and approximately $2.0x10^6$ spores/mL for *G. roseum* and washed and unwashed conidia of *M. verrucaria*. Fifty microlitres of spore suspension was spread over cellophane strips on four different agars (V-8, CD, PDA and 1%). Treatments included *A. brassicae* in combination with *G. roseum* isolate 98 or washed or unwashed conidia of *M. verrucaria*. *Alternaria brassicae, G. roseum* and washed and unwashed *M. verrucaria* conidia, respectively, served as controls. Two plates of each type of agar were observed at each time for treatments and controls. As the two isolates of *G. roseum* behaved similarly with respect to time when parasitism began and also in the mode of parasitism, only *G. roseum* isolate 98 was used in this experiment. Due to space restrictions in the incubator, only one combination of *A. brassicae* plus an antagonist could be observed at any one time. Plates were incubated in the dark at 23 ± 2°C. Cellophane strips were removed from plates and slides were made as previously described after 39, 42, 45 and 48 hours of incubation. Mycelial growth of the fungi, percent of conidia parasitized and severity of parasitism were recorded. In order to accurately assign numeric values to mycelial growth, 48 hour slides were observed first and 39 hour slides were observed last. In so doing, it was possible to observe the maximum growth of each fungus first and rate its growth over time more accurately. Severity of parasitism (sop) was rated on a scale of 0 to 5. If there was no coiling of the antagonist around the *A. brassicae* conidium, a rating of 0 was given, 1% to 20% was rated as 1, 21% to 40% was rated as 2, 41% to 60% was rated as 3, 61% to 80% was rated as 4 and 81% to 100% was rated as 5. The percentages refer to the visible area of the conidium that was covered by the antagonist.

Fluorescence Microscopy

Fungi were grown on cellophane strips on 1% water agar and observed at time zero and after 39 and 48 hours of incubation. The concentration of spore suspensions used for this experiment were the same as those used for the parasitism experiment. Fifty microlitres of spore suspension was applied to cellophane on 1% water agar. At each observation time, five slides of each treatment were observed and the characteristics of conidia and mycelia on each strip were recorded. Stock and working solutions of fluorescein diacetate (FDA) were 2 mg/mL in acetone kept at -18°C and 10 or 20 µg/mL in 60 mM phosphate buffer (pH 7.5). Stock and working solutions of propidium iodide (PI) were 2 mg/mL and 150 or 300 µg/mL in the same phosphate buffer. Dyes were applied in combination. Higher concentrations of dye were used for time zero observations because upon addition of the dye solution to the spore suspension, the dye solution was diluted. Lower concentrations of dye were used at the later observation times because the water used to make the conidial suspension had dried and dilution was no longer a factor in determining the concentration of the dyes. Fifty microlitres of dye solution was applied to each slide which was then covered with a cover slip and observed with a Zeiss Universal Microscope equipped with an excitation filter of 450-490 nm (blue light) and a barrier filter of 520 nm (Firstencel et al., 1990). Theoretically, green fluorescence indicates viability while orange fluorescence indicates lack of viability.

Treatments included combinations of *A. brassicae* with *M. verrucaria* washed and unwashed conidia, metabolites of *M. verrucaria* and conidia of isolate 98 of *G. roseum*. Controls consisted of each fungus on its own. One percent water agar plates were used as it was easiest to determine the interactions on this medium. Due to space restriction in the incubator, one *A.*

brassicae plus antagonist combination was observed at each time. Once again, only *G. roseum* isolate 98 was used. As overall controls, freshly harvested and autoclaved conidia of each fungus were observed. As well, all treatments and controls were observed with buffer only.

Scanning and Transmission Electron Microscopy

Specimens used for scanning electron microscopy (SEM) were grown on cellophane strips on 1% water agar. Small pieces of cellophane were cut, mounted on a cryo-stub, frozen in a nitrogen slush, sublimed, coated with gold and observed on the cryostage of a Cambridge Stereoscan 250 scanning electron microscope. For those specimens which were freeze-fractured, the specimen on the cryo-stub was immersed in a nitrogen slush, frozen and scored with a razor blade prior to sublimation.

For transmission electron microscopy (TEM) fungi were grown on cellophane strips on 1% water agar, brushed off the cellophane into 3% glutaraldehyde in phosphate buffer and fixed for 18 hours. Fungi were then washed twice in phosphate buffer (pH 7.0) and fixed in 2% osmium tetroxide in phosphate buffer for 4 hours, dehydrated in a graded water-ethanol series, infiltrated with propylene oxide and embedded in Spurr's Resin. Thick (approximately 1 µm) and ultrathin (approximately 80 - 90 nm) sections were cut on a Reichert OMU2 microtome using glass and diamond knives, respectively.

Thick sections were placed on gelatin coated slides (250 mL distilled water, 1.25 g gelatin, 0.125 g $CrK(SO_4)_2.12H2O$), stained with toluidine blue for 2 minutes and mounted in either Permount or DPX mountant under a #1 glass cover slip.

The ultrathin sections were stained with saturated uranyl acetate for 1 hour and Sato's lead citrate for 2 minutes on 300 mesh copper grids and observed in a Philips EM 201 transmission electron microscope.

Statistical Analyses

Analysis of variance was used in determining the differences between treatments and was computed using GLM on SAS. A least significant difference procedure (t test) was used. An alpha of 0.05 was used to decide if the differences were significant.

Results

Germination Studies

The effects of the treatments were most apparent after 8 hours. Figure 3.1 shows the germination of *A. brassicae* spores under different treatments as compared to the *A. brassicae* control. The *G. roseum* treatment and the three *M. verrucaria* treatments had no affect on germination of *A. brassicae* when they were used at a concentration of 10^4 spores/mL. At a concentration of 10^5 spores/mL the only treatment that affected *A. brassicae* germination was unwashed conidia of *M. verrucaria*, however the inhibition of germination was not drastic. Washed conidia of *M. verrucaria* and metabolites of *M. verrucaria* slightly inhibited *A. brassicae* germination when used at a concentration of 10^6 spores/mL. The germination of *A. brassicae* conidia was drastically inhibited by unwashed conidia of *M. verrucaria* at a concentration of 10^6 spores/mL. *Gliocladium roseum* isolate 98 did not inhibit *A. brassicae* spore germination even when used at a concentration of 10^6 spores/mL.

Figure 3.2 shows the overall trend of *A. brassicae* spore germination over 8 hours. The pattern of *A. brassicae* germination was not altered by either washed *M. verrucaria* conidia or by *G. roseum* isolate 98 conidia. Metabolites of *M.verrucaria* used at their highest concentration (10^6) inhibited germination of *A. brassicae* at all observation times. Germination of *A. brassicae* was inhibited by the presence of unwashed conidia of *M. verrucaria* at all times when unwashed conidia of *M. verrucaria* were used at concentrations of 10^5 spores/mL and 10^6 spores/mL.

After 6 hours, when washed conidia of *M. verrucaria* at a concentration of 10^4 spores/mL were associated with *A. brassicae*, germination of *M. verrucaria* was enhanced (Figure 3.3). At concentrations of 10^5 spores/mL and 10^6 spores/mL the germination of washed *M. verrucaria* conidia was inhibited when combined with *A. brassicae.* After 8 hours, the differences in percent germination of washed conidia of *M. verrucaria* alone and in combination with *A. brassicae* were not as great for concentrations of 10^4 and 10^6 spores/mL. The same general trends were seen with the unwashed conidia of *M. verrucaria* in association with *A. brassicae* (Figure 3.4). The germination rates of *G. roseum* isolate 98 conidia were greatly affected by the presence of *A. brassicae* (Figure 3.5). After 6 hours, the germination rates were significantly higher for *G. roseum* in association with *A. brassicae.* However, with the exception of the concentration of 10^4 spores/mL the germination rates were similar after 8 hours.

Germ tube length of *A. brassicae* (Figures 3.6 - 3.12) was affected differently by the various *G. roseum* and *M. verrucaria* treatments. Average germ tube lengths after 8 hours growth for *A. brassicae* control and when combined with *G. roseum* at concentrations of 10^6, 10^5 and 10^4 spores/mL were 111.8, 108.6, 109.9 and 104.3 µm respectively. The range of germ tube lengths for the same treatments were 6.0-255.0, 6.0-318.0, 9.0-273.0 and 3.0-270.0 µm respectively. Washed conidia of *M. verrucaria* conidia caused a slight reduction in germ tube length with increasing concentration. Average germ tube lengths for *A. brassicae* control and when combined with washed *M. verrucaria* conidia at concentrations of 10^6,10^5 and 10^4 spores/mL were 111.7, 80.6, 97.2 and 107.9 µm respectively with the range of germ tube lengths for the same treatments being 9.0-330.0, 6.0-225.0, 9.0-243.0 and 3.0-234.0 µm respectively. Unwashed conidia of *M. verrucaria* had the greatest affect upon germ tube lengths. The average germ tube lengths for control and unwashed *M. verrucaria* conidia combined with *A. brassicae* in the same order as previously mentioned are 123.8, 35.4, 64.2 and 117.7 µm. The range of germ tube lengths for the same treatments in the same order are 9.0-330.0, 3.0-156.0, 3.0-225.0 and 6.0-285.0 µm. The same trend was seen when *A. brassicae* was combined with metabolites of *M. verrucaria*. Average germ tube lengths for *A. brassicae* control and *A. brassicae* combined with metabolites of *M. verrucaria* in the same order of concentrations as previously mentioned are 108.8, 59.2, 85.3 and 91.2 µm. The range of germ tube lengths for the same treatments in the same order are 6.0-265.0, 3.0-249.0, 3.0-240.0 and 6.0-273.0 µm.

The percentage of cells of *A. brassicae* conidia which germinated was not as greatly affected by the treatments as were the germ tube lengths. For *A. brassicae* controls and most treatments on average between 20% to 30% of cells germinated after 8 hours. When *A. brassicae* conidia were combined with unwashed conidia of *M. verrucaria* at a concentration of 10^6 spores/mL the average percentage of cells that germinated was 1.6.

Colony Interactions

Five Days Growth

Sporulation of *M. verrucaria* or either isolate of *G. roseum* was not evident in any of the treatments. On both 1% water agar and CD there was no inhibition of *A. brassicae* by any of the antagonists. When the fungi were grown on PDA, *A. brassicae* growth was slightly inhibited by both isolates of *G.*

roseum, while it was markedly inhibited by *M. verrucaria* (Figure 3.13). When observing colony growth on V-8, no inhibition of *A. brassicae* was evident with either isolate of *G. roseum*, but slight inhibition of *A. brassicae* occurred when *M. verrucaria* was present. Intermingling of fungi was apparent on both 1% water agar and V-8.

Ten Days Growth

No distinct pattern of antagonist sporulation was apparent after ten days growth. *Gliocladium roseum* sporulated on V-8 only where it was overgrowing *A. brassicae* colonies. *Myrothecium verrucaria* did not sporulate on CD, sporulated sparsely on 1% water agar and sporulated very heavily on PDA and V-8. Some inhibition of *A. brassicae* was evident on 1% water agar when grown in combination with *M. verrucaria*. *Alternaria brassicae* on PDA was slightly inhibited by both isolates of *G. roseum* (Figure 3.14), but was severely inhibited by *M. verrucaria* (Figure 3.13). This inhibition occurred without contact between the two colonies. *Gliocladium roseum* grew over the *A. brassicae* colonies on PDA. When observing interactions on CD, there was no evidence of inhibition of *A. brassicae* by any of the parasites. Both isolates of *G. roseum* grew over the *A. brassicae* colonies, while *M. verrucaria* grew around the *A. brassicae* colonies. When grown on V-8, *A. brassicae* was slightly inhibited by all antagonists. Both isolates of *G. roseum* were observed growing over *A. brassicae* and sporulated in that area only. *Myrothecium verrucaria* sporulated profusely except where it grew over the *A. brassicae* colony. However, a similar pattern of sporulation was seen in *M. verrucaria* control plates.

Fifteen Days Growth

On 1% water agar *M. verrucaria* and both isolates of *G. roseum* sporulated sparsely while *A. brassicae* did not. The *M. verrucaria* colony sporulated more on the side that was away from the *A. brassicae* colony. There was sparse mycelial growth of *A. brassicae* and both isolates of *G. roseum* and in both cases, the *A. brassicae* colony was almost totally overgrown by *G. roseum*. The colonies of *M. verrucaria* and *G. roseum* both exhibited sparse mycelial growth which intermingled. Colonies of both isolates of *G. roseum* grew over *A. brassicae* colonies on PDA. *Alternaria brassicae* sporulated, however, both isolates of *G. roseum* did not sporulate. On PDA, the *M. verrucaria* colony grew over the *A. brassicae* colony. Both fungi sporulated heavily, but sporulation of *M. verrucaria* was more pronounced on the side of the colony that was away from *A. brassicae*. *Alternaria brassicae* colonies were completely grown over by both isolates of *G. roseum* on CD. Both isolates of *G. roseum* sporulated in older areas. When *M. verrucaria* and *A. brassicae* were observed in co-culture on CD there was dense mycelial growth of *M. verrucaria*, but it did not sporulate. There was dense sporulation of *A. brassicae*. The colony of *M. verrucaria* grew around the colony of *A. brassicae*. When observed on V-8 agar, *G. roseum* isolates 98 and 17 sporulated profusely in older areas of the colony and in areas of the colony that grew over the *A. brassicae* colony which sporulated heavily. *Myrothecium verrucaria* colonies grew over *A. brassicae* colonies and sporulated densely except where they grew over *A. brassicae* which showed dense sporulation. The *M. verrucaria* colony produced a profuse amount of exudate.

Observation of Slides

Very little parasitism of *A. brassicae* by *M. verrucaria* occurred. In contrast, *G. roseum* parasitized the hyphae of *A. brassicae* on PDA only (Figure 3.15). The parasitized hyphae appeared to be disintegrated.

Parasitism Studies

After 39 hours of incubation, mycelial growth of *G. roseum* isolate 98 when in combination with *A. brassicae* was greater than that of *G. roseum* on its own. At the later observation times there was not a marked difference in the growth of *G. roseum* isolate 98 whether it was in combination with *A. brassicae* or not. The mycelial growth of *A. brassicae* was slightly less in combination with *G. roseum* isolate 98 than the *A. brassicae* control. When *A. brassicae* was combined with washed and unwashed conidia of *M. verrucaria*, the resulting growth was inhibited when compared to the *A. brassicae* control. When the mycelial growth resulting from washed and unwashed conidia of *M. verrucaria* was observed, it was greater when in combination with *A. brassicae* on CD than by itself, but the comparison was about the same on the other media. The mycelial growth resulting from *A. brassicae* conidia and washed conidia of *M. verrucaria* was lower on CD and 1% water agar than on the other media. This was also true for the *A. brassicae*-unwashed conidia of *M. verrucaria* combination and the *A. brassicae*-*G. roseum* isolate 98 combination.

Gliocladium roseum isolate 98 (Figures 3.16-3.18)

After 39 hours of incubation, sop was greatest on V-8 agar, followed by CD and PDA. Parasitism severity was least on 1% water agar. V-8 and PDA showed the greatest sop at 42 hours followed by CD and 1% water agar. The sop at 45 hours was greatest on CD, followed by PDA, V-8 and lastly 1% water agar. The same trend was seen after 48 hours of incubation as after 45 hours of incubation.

In the case of 1% water agar, sop was not significantly different after 39 and 42 hours of incubation. Severity of parasitism was also not significantly different between 45 and 48 hours, however, sop increased dramatically between the observations made at 42 and 45 hours.

There was no significant difference in sop on CD at 39 and 42 hours. Parasitism severity was significantly greater at 45 hours compared to the previous observation times and again at 48 hours. At both 45 and 48 hours, sop was greatest on CD.

For both PDA and V-8, at 42 hours, sop was significantly different from that at 39 hours. At 42 and 45 hours, there was no significant difference. Fourty-eight hours after incubation, sop was significantly greater than at all other observation times.

Unwashed *M. verrucaria* (Figures 3.19-3.21)

After 39 hours of incubation, sop was least on CD, but was significantly higher on 1% water agar. At 39 hours, parasitism was most severe on PDA and V-8. After 42 hours incubation, sop was the same for CD and 1% water agar. It was significantly higher on V-8 and was most severe on PDA with PDA being significantly different from all the other media at 42 hours. Severity of parasitism at 45 hours was least on V-8 and greatest on PDA. Both CD and 1% water agar showed greater parasitism than V-8 but less than PDA. Parasitism severity was least on V-8 at 48 hours and greatest on PDA. There was no significant difference between sop on 1% water agar, CD and PDA, but all were significantly greater than V-8.

For all media sop increased with time. With the exception of V-8, sop was significantly greater at each observation time for each agar when compared with itself. Parasitism severity on V-8 was significantly greater at 42 hours than at 39 hours and at 45 hours than at 42 hours, but was not significantly different between 45 and 48 hours.

Washed *M. verrucaria* (Figures 3.22-3.24)

After 39 hours of incubation, sop was greatest on PDA, followed by CD, V-8 and lastly 1% water agar. When sop was observed at 42 hours, PDA and CD showed the same degree of sop and both were greater than V-8 and 1% water agar, which showed the same degree of sop. PDA showed the most marked sop at 45 and 48 hours, followed by CD and lastly V-8 and 1% water agar which showed the same sop.

For all media, the sop increased over time. When each medium was compared to itself over time, the same pattern was seen for all media. Parasitism severity increased significantly between 39 and 42 hours and again between 42 and 45 hours, but levelled off at that point and no further increase in sop was seen at 48 hours.

Percentage of *A. brassicae* Conidia Parasitized

The percentage of *A. brassicae* conidia parasitized by *G. roseum* and *M. verrucaria* increased with time on all media (Table 3.1). *Myrothecium verrucaria* grown from washed conidia and *G. roseum* both parasitized fewer conidia on 1% water agar than on the other media used. This was not the case for *M. verrucaria* grown from unwashed conidia. In this instance, after 39 and 42 hours of incubation, the percentage of *A. brassicae* conidia parasitized was least on CD, but after 45 and 48 hours of incubation, percentage of *A. brassicae* conidia parasitized was least on 1% water agar. For the high nutrient media, the percentage of *A. brassicae* conidia parasitized was similar for all host-antagonist combinations.

Fluorescence Microscopy

The conidia of *A. brassicae*, *G. roseum* isolate 98 and *M. verrucaria* (washed and unwashed) all fluoresced green (indicating viability) when a fresh spore suspension was observed on a glass slide with no cellophane. Autoclaved conidia of all fungi fluoresced orange (indicating lack of viability) when observed on a glass slide with no cellophane. This indicated that the fluorescent dyes FDA and PI were effective in differentiating between living and dead conidia, respectively.

When the different combinations of fungi and their controls were observed with no fluorescent dyes (buffer only), some very faint autofluorescence of hyphae was observed. After 39 and 48 hours of incubation, *A. brassicae* hyphae fluoresced faintly green. If there was a mass of *A. brassicae* hyphae, there was a mixture of faint green and faint orange fluorescence. The hyphae of the other fungi occasionally fluoresced faint green. At time zero, some of the conidia of *G. roseum* isolate 98 fluoresced faint green. Faint orange fluorescence of a young *A. brassicae* conidium was also observed. Neither washed or unwashed conidia of *M. verrucaria* fluoresced.

When observed with fluorescent dyes, at time zero, most cells of *A. brassicae* conidia exhibited green fluorescence, however a few cells fluoresced orange. Those conidia which were younger or lighter colored showed more intense and more stable fluorescence (the green color did not fade quickly). Some hyphal fragments of *A. brassicae* were present at time zero and most of these fragments fluoresced bright green. Segments which were narrower fluoresced bright orange. A few cells of the conidia of *A. brassicae* fluoresced green after 39 hours. These cells were usually germinated. Few cells of the conidia fluoresced orange. Again, most hyphae fluoresced green, while a few narrow segments fluoresced orange. Darker colored, older conidia fluoresced faintly or not at all. Similar observations were made at 48 hours (Figure 3.25).

At time zero, the washed conidia of *M. verrucaria* did not fluoresce. When observed after growing for 39 hours, the hyphae fluoresced faint to bright orange, while the conidia did not fluoresce. After 48 hours some green hyphae were seen but most hyphae were orange. Again, conidia did not fluoresce.

Unwashed conidia of *M. verrucaria* did not fluoresce at any of the observation times. At 39 hours the hyphae fluoresced green and orange, but the majority of fluorescence was orange. Similar observations were made at 48 hours.

For both *M. verrucaria* treatments, hyphal fluorescence varied from slide to slide. The hyphae on some slides exhibited more green fluorescence than the hyphae on other slides and vice versa for orange fluorescence.

Conidia of *G. roseum* isolate 98 fluoresced faint green or faint orange at time zero. When observed after 39 hours, most conidia fluoresced green, but some did fluoresce orange. Most of the hyphae observed at 39 hours were fluorescing green, although some were fluorescing orange. Similar traits were observed at 48 hours.

When combinations of fungi were observed at time zero the results were the same as for each of the fungi in isolation. When *A. brassicae* was in combination with *M. verrucaria* conidia, the hyphal growth of *A. brassicae* was reduced.

After 39 hours, when *A. brassicae* was in combination with hyphae grown from washed conidia of *M. verrucaria*, the hyphae of *A. brassicae* fluoresced faint to intense orange and some cells of the conidia fluoresced orange. These orange cells were usually associated with *M. verrucaria* hyphae. No green fluorescence of *A. brassicae* was observed. The hyphae of *M. verrucaria* were mostly orange, but a few green hyphae were present. *Myrothecium verrucaria* hyphae associated with *A. brassicae* conidia fluoresced orange. The same observations were made at 48 hours (Figure 3.26)

When the *A. brassicae*-unwashed *M. verrucaria* combination was observed after 39 hours, some cells of *A. brassicae* conidia fluoresced orange and were again in association with *M. verrucaria* hyphae. There were a few *A. brassicae* hyphae that fluoresced green, but most fluoresced orange and were associated with *M. verrucaria* (Figure 3.27). The hyphae of *M. verrucaria* fluoresced green and orange. The same observations were made at 48 hours (Figure 3.28).

Most of the cells of the conidia of *A. brassicae* combined with metabolites of *M. verrucaria* fluoresced green, however a few did fluoresce orange. There was more orange fluorescence of the hyphae produced by *A. brassicae* when in combination with metabolites of *M. verrucaria* (Figure 3.29) than with the *A. brassicae* control. These observations were the same at 39 and 48 hours.

Once again at 39 hours, *A. brassicae* cells in association with antagonist hyphae fluoresced orange. Some cells of *A. brassicae* conidia fluoresced orange but were not associated with antagonist hyphae. A few cells in the conidia fluoresced green. In this case the antagonist was *G. roseum* isolate 98. The hyphae of *A. brassicae* fluoresced both green and orange. The majority of *G. roseum* isolate 98 hyphae fluoresced green as did the conidia, however, some of the hyphae associated with *A. brassicae* conidia fluoresced orange (Figure 3.30). The same observations were made at 48 hours.

Scanning and Transmission Electron Microscopy

The interactions of *M. verrucaria* and *G. roseum* with *A. brassicae* were remarkably similar. Both *M. verrucaria* and *G. roseum* coiled around the conidia of *A. brassicae* (Figures 3.31 and 3.32). The hyphae of both *M. verrucaria* and *G. roseum* appeared to penetrate the conidia of *A. brassicae* (Figures 3.31 and

3.32). No appressoria were produced by either *M. verrucaria* or *G. roseum*. The appearance of mucilage or any special substances produced to help the antagonist attach to *A. brassicae* were also lacking in these host-antagonist systems. However, this may have been a result of the fixation technique used.

Hyphae of *M. verrucaria* penetrating the conidia of *A. brassicae* and growing within the conidia of *A. brassicae* were observed when thick sections were observed with an oil immersion lens (Figure 3.33). When observed with the transmission electron microscope, *M. verrucaria* was found penetrating the conidia of *A. brassicae* but no reaction zones were observed (Figure 3.34). The walls of the *A. brassicae* conidium were pushed inward in addition to being partly dissolved.

Gliocladium roseum was found to be growing inside the conidia of *A. brassicae* (Figure 3.35). A perforation in the conidium of *A. brassicae* was seen where *G. roseum* hyphae had been dislodged from the point of penetration. The cell wall of *A. brassicae* was dissolved by *G. roseum* at the point of penetration. There was no indentation of the cell wall, thus there was no evidence of mechanical penetration (Figures 3.36 and 3.37).

Discussion

Germination Studies

The inability of *G. roseum* isolate 98 to affect *A. brassicae* in any of the ways observed in the germination studies indicates that this isolate of *G. roseum* would not be effective in preventing the onset of disease but may be useful for slowing the progression of disease caused by *A. brassicae*. Although several species of *Gliocladium* have been reported to produce inhibitory compounds it appears that this isolate of *G. roseum* does not produce any. That is the most likely reason for *A. brassicae* not being affected by this isolate of *G. roseum*. It must be remembered, however, that the conidia of *G. roseum* were washed and what few inhibitors the conidia may have been carrying would most likely have been washed off. However, in initial studies with unwashed conidia of *G. roseum* , no affects upon germination or growth of *A. brassicae* were noted.

The most reasonable explanation for *A. brassicae* to be even slightly inhibited by the three different *M. verrucaria* treatments is that *M. verrucaria* is known to produce secondary metabolites which are potent inhibitors of protein synthesis. The reduced action of the washed conidia of *M. verrucaria* in comparison to that of unwashed conidia of *M. verrucaria* is due to the fact that the majority of the inhibitory metabolites were removed from the conidia when they were washed. It is possible that the effects of washed conidia of *M. verrucaria*, although slight, were due to a small amount of metabolite being carried on the conidia and the production of metabolites through the metabolic activities of the conidia. However, the amount of metabolites produced through metabolic activities over a period of 8 hours is not likely to be enough to significantly affect *A. brassicae*. The isolated crude metabolites of *M. verrucaria* affected *A. brassicae* more than the washed conidia of *M. verrucaria* but less than the unwashed conidia of *M. verrucaria*. A possible explanation for this is that the concentration of metabolites of *M. verrucaria*, although less dilute than the metabolites carried on unwashed conidia, were more concentrated than those carried on the washed conidia.

As with the other concentrations of *M. verrucaria* treatments used, the effects of unwashed *M. verrucaria* conidia on *A. brassicae* increased with increasing concentration. The almost complete inhibition of growth seen with the unwashed conidia of *M. verrucaria* at the highest concentration used indicates the potency of the metabolites produced by this fungus. The trichothecenes, the family of toxins to which the metabolites of *M. verrucaria* belong, are inhibitors of eukaryotic protein synthesis (Beremand and McCormick, 1992; Desjardins, 1992; Ueno, 1983). The basis of the inhibition of protein synthesis occurs via binding to the 60S ribosomal subunit (Beremand and McCormick, 1992; Desjardins, 1992). Due to the degree and type of oxidations and esterifications at different positions, the trichothecenes vary in toxicity and their ability to effectively inhibit protein synthesis. It is these variations which are responsible for the diversity and number of trichothecenes identified (over 90 in 1992) (Beremand and McCormick, 1992). T-2 toxin and 4,15-diacetoxyscirpenol are among the most toxic and inhibit initiation of protein synthesis (Desjardins, 1992). Trichothecenes such as 15-deacetylcalonectrin (which is an example of the less-oxygenated trichothecenes) are less toxic and inhibit the elongation and termination of protein synthesis rather than its initiation (Desjardins, 1992). The trichothecenes may also block DNA synthesis (Hesseltine, 1983).

Germination of the washed and unwashed conidia of *M. verrucaria* behaved similarly after 8 hours, but not after 6 hours. After 6 hours, the washed conidia of *M. verrucaria* at the higher concentrations when in combination had a low germination rate in comparison with the control for washed conidia of *M. verrucaria*. This may have been due to competition for nutrients from the media or perhaps *A. brassicae* produced some metabolite which affected the growth of the conidia up to that time. The germination of unwashed conidia of the *M. verrucaria* control and when in combination with *A. brassicae* were similar after 6 hours.

Pachenari and Dix (1980) reported that when *G. roseum* was grown on *Botrytis allii* mycelium, the spores of *G. roseum* were stimulated. This was also the finding when the germination of *G. roseum* isolate 98 conidia were observed after 6 hours. One possible explanation for this is that *A. brassicae* may have leaked nutrients which could be used by *G. roseum*, thereby increasing its germination rate in response to an exogenous nutrient source.

Colony Interactions

Three outcomes are possible when interactions occur between mycelial systems. Firstly, there may be neutral intermingling of hyphae. Secondly, a situation known as deadlock, where the mycelium of either fungus does not enter the territory occupied by the other fungus, may occur. Lastly, a situation referred to as invasion of domain, where the mycelium of one fungus may partially or completely replace the other, may occur (Rayner and Boddy, 1988). All three of these interactions occurred in the colony interaction studies. Neutral intermingling was most common on 1% water agar. This may be due to the low nutrient status of the medium which would result in the growth of less vigorous colonies which may not have the "energy" to overgrow their host colonies. Stahl and Christensen (1992) determined that conditions which favor sparse mycelial development appear to facilitate neutral intermingling of hyphae. Stahl and Christensen (1992) observed that more territorial behavior, such as invasion of domain and deadlock occurred on high nutrient media than on low nutrient media. The situation known as deadlock occurred when *M. verrucaria* and *A. brassicae* were grown in co-culture on CD. All other interactions involved invasion of domain where the *A. brassicae* colonies were either partially or completely overgrown by *M. verrucaria* or either isolate of *G. roseum.* A possible explanation for the occurrence of deadlock is that due to the constituents of the medium or the size of the metabolite molecules, the metabolites of *M. verrucaria* may not have been able to move through the medium to the *A. brassicae* colony and thus the *A. brassicae* colony would not have been weakened. This may result in more waste products of *A. brassicae* being produced and inhibiting the approach of *M. verrucaria*. Lack of sporulation of *M. verrucaria* on CD may have resulted in less metabolite production. Thus, *A. brassicae* may not have been inhibited possibly resulting in the inability of *M. verrucaria* to overgrow *A. brassicae*. Invasion of domain was the most common type of colony interaction observed and it occurred only on the high nutrient media. This is most likely because the colonies of *M. verrucaria* and *G. roseum* were growing vigorously and therefore had more "energy" to overgrow the colonies of *A. brassicae*. This indicates that in order for *A. brassicae* to be parasitized by *G. roseum* or *M. verrucaria,* a high nutrient food source may be required.

Contradictory reports exist explaining the lack of parasitism seen on high and low nutrient media. In these studies, results to support both reports were obtained from the same experiment. The lack of parasitism of *A. brassicae* by *M. verrucaria* and *G. roseum* on 1% water agar was probably due to the *M. verrucaria* and *G. roseum* hyphae being weakened by the low nutrient status of the medium. On the higher nutrient media the lack of parasitism may be due to *M. verrucaria* and *G. roseum* growing saprophytically on the media as opposed to parasitizing *A. brassicae* in order to obtain nutrients. In general, members of the genus *Myrothecium* are saprophytic. On PDA, however, *A. brassicae* was severely parasitized by *G. roseum*. One possible explanation for this is that the nutrient sources in PDA are adequate for the growth and sporulation of *G. roseum* but if a more balanced nutrient source, such as *A. brassicae*, is present then *G. roseum* will parasitize *A. brassicae* instead of growing saprophytically. The hyphae of *A. brassicae* when parasitized by *G. roseum* appeared to be disintegrated. This observation is similar to that made by Moody and Gindrat (1977) who reported that mycelial mats of *P. sclerotioides* were nearly completely destroyed and disintegrated by *G. roseum* after one week of incubation.

Parasitism Studies

Butler (1957) determined that when conditions result in poor vegetative growth of either the host or parasite, there is a reduction in the amount of infection of the host. This indicates that good growth of the host and parasite are a prerequisite for parasitism. Growth of *A. brassicae*, *M. verrucaria* and *G.*

roseum were all reduced on 1% water agar when compared to the other media. This may explain the reduced sop seen on 1% water agar when compared to the high nutrient media. However, in all treatments with *M. verrucaria*, growth of *A. brassicae* was inhibited but parasitism was severe nonetheless.

The differences in sop are most likely due to the differences in nutrient composition of the media and the differing nutritional needs of *M. verrucaria* and *G. roseum*. If the carbon or nitrogen sources present in the media were not satisfactory for the growth of the antagonist, it is likely that parasitism of *A. brassicae* would be more severe.

Unlike in the colony interaction studies, parasitism of *A. brassicae* by *M. verrucaria* and *G. roseum* occurred on all media. This suggests that application of conidia of *M. verrucaria* and *G. roseum* to *A. brassicae* conidia is more efficacious than hyphae from an established culture. The hyphae arising from the conidial suspension may have been more vigorous than those resulting from colony growth. As well, many more metabolic waste products would have been present in the colony interaction studies than in the parasitism experiments. As a result, fungi in the parasitism experiments would have had better conditions for growth than fungi in the colony interaction studies resulting in increased parasitism of *A. brassicae*.

Another interesting difference between the colony interaction studies and the parasitism studies was that deadlock occurred between colonies of *A. brassicae* and *M. verrucaria* on CD while in the parasitism studies, there was intermingling of the fungi (although *A. brassicae* growth was inhibited) on CD. Stahl and Christensen (1992) observed the same phenomenon when *Penicillium restrictum* was paired with itself. Intermingling was present on an agar coated slide while deadlock occurred in plate cultures. These findings were attributed to age differences of the fungi on slide cultures and plate cultures.

Fluorescence Microscopy

In theory, live (viable) specimens fluoresce green and dead (non-viable) specimens fluoresce orange when stained with fluorescein diacetate (FDA) and propidium iodide (PI), respectively. FDA is taken in by all cells, but hydrolyzed only by live cells (Alvarez et. al, 1991). PI is generally used in studies involving nuclear materials (Eilam et al, 1992; Shapiro, 1988) but has been used in viability studies (Firstencel et al., 1990). The most puzzling of the results obtained through fluorescence microscopy were those of the orange fluorescence of the mycelia of *M. verrucaria* and the lack of fluorescence of the conidia of this fungus. The organelles and hyphal walls fluoresced brilliant orange, indicating death of this fungus if interpreted in the intended manner. However, it is possible that the orange fluorescence produced masked any green fluorescence emanating from the mycelia of this fungus. It is also possible that PI was acting more as an indicator for the presence of DNA in the nuclei and mitochondria than as an indicator of viability. Another possibility is that the concentration of FDA used was not suitable to induce fluorescence of *M. verrucaria*. A possible explanation for the lack of fluorescence of *M. verrucaria* conidia may be that the metabolites carried on the conidia blocked the fluorescence of the dyes. This is probably not the cause of lack of fluorescence because both washed and unwashed conidia of *M. verrucaria* did not fluoresce. The conidia of *M. verrucaria* are hyaline, so the lack of fluorescence cannot be attributed to dark coloration. Even though the conidia are hyaline, they may be thick walled, thus impeding the entry of the stain into the cells or masking any fluorescence which may have occurred. Barak and Chet (1986) reported that the conidia of *Trichoderma hamatum* did not fluoresce in the presence of FDA, indicating that the results obtained here are not an isolated incident.

Other factors which may have affected the results obtained are the length of the incubation period in the dyes and the wall structure of the fungi involved. Incubation time should be considered as a factor in fluorescence studies. However, in this situation it is unlikely that incubation period in the dye had an affect on the fluorescence observed. This is because there was very little variation observed between slides which had been incubated for differing lengths of time. It may be possible that differences in the wall structure of the fungi may have eased or impeded the entry of the dyes, thereby affecting the results.

Many of the cells of *A. brassicae* conidia associated with either *G. roseum* or *M. verrucaria* fluoresced orange. This fluorescence may have been an indicator of the viability of the cells or an indicator of the presence of the antagonist within the cells or coiling around the cells of *A. brassicae*. When observed with tungsten light, the cells of *A. brassicae* conidia which fluoresced orange were all associated with either *M. verrucaria* or *G. roseum*. Thus, the orange fluorescence of these conidial cells was probably due to a combination of the three previously mentioned factors. All antagonist hyphae associated with *A. brassicae* conidia fluoresced orange and if a mass of hyphae were inside the conidial cells of *A. brassicae* the cells would most likely fluoresce orange. Hyphae of *A. brassicae* which were parasitized usually fluoresced orange, indicating death of these hyphae. This coincides with the findings of Barak and Chet (1986) in which fungal viability during mycoparasitism was observed using FDA. They found that parasitized hyphae of *Rhizoctonia solani* did not fluoresce, while hyphae that were not parasitized did fluoresce.

Fluorescence microscopy was plagued with problems. The most notable of which were the lack of fluorescence of *M. verrucaria* conidia, the orange fluorescence of hyphae assumed to be alive and the rapid fading of green fluorescence. The pH, concentrations of stains and the molarity of buffer used were chosen because this protocol successfully induced fluorescence of *A. brassicae*. It may be that these concentrations were not optimal for *M. verrucaria* or *G. roseum*. Different protocols are required for different fungi depending on wall thickness and coloration of the fungal structures being observed. Firstencel et al. (1990) reported that within a genus different protocols were required for different species. As well, different protocols are required for different spore types (Firstencel et al., 1990). This may help to explain the lack of fluorescence of *M. verrucaria* conidia while *A. brassicae* conidia fluoresced.

Scanning and Transmission Electron Microscopy

Both *M. verrucaria* and *G. roseum* coiled around the conidia of *A. brassicae*. The hyphae of both antagonists appeared to penetrate the conidia at certain points. At these points, short branches appeared to be formed by *M. verrucaria* and *G. roseum*. It may be that these branches behaved similarly to appressoria and may have aided in the penetration (Baker, 1990) of *A. brassicae* conidia. *Gliocladium roseum* has been reported to form specialized penetrating organs (Walker and Maude, 1975).

Upon light microscope observation of thick sections of *A. brassicae* conidia combined with either *M. verrucaria* or *G. roseum*, hyphae of the antagonists were seen inside the conidia of *A. brassicae*, confirming that the antagonists were penetrating the conidia of *A. brassicae*. When observed with the aid of a transmission electron microscope, *M. verrucaria* was found penetrating the walls of *A. brassicae* conidia. The walls of the conidia were pushed inwards and partially degraded suggesting a combination of mechanical and enzymatic penetration.

With the aid of the scanning electron microscope, penetration of the walls of *A. brassicae* by *G. roseum* was observed. The walls of *A. brassicae* were not pushed inward but were cleanly perforated. This suggested that *G. roseum* entered the conidia of *A. brassicae* by enzymatic means only. *Gliocladium roseum* has been reported to produce both chitinolytic and *B*-1,3-glucocanolytic compounds (Di Pietro et al., 1993; Pachenari and Dix, 1980).

Conclusions

The metabolites produced and carried by the conidia of *M. verrucaria*, when present in sufficient quantities, are very effective in reducing the germination and growth of *A. brassicae*. The effects of these metabolites were also apparent in both colony interaction studies and parasitism studies. The results obtained in the colony interaction studies indicate that in most situations when a high nutrient food source is available and *M. verrucaria* and *G. roseum* are growing well in colonies in petri plates, very little parasitism of *A. brassicae* occurs. As well, when grown on 1% water agar, the fungi are too weak to parasitize *A. brassicae*. When applied as a conidial suspension, *M. verrucaria* and *G. roseum* were effective antagonists of *A. brassicae* on all media studied. The increased sop noted on the high nutrient media indicated that both *M. verrucaria* and *G. roseum*, when applied as a conidial suspension, are more effective antagonists when a high nutrient food source is present. If a satisfactory protocol could be developed, fluorescence microscopy could be a very valuable tool in determining the viability of parasitized *A. brassicae* conidia. Through scanning and transmission electron microscopy it was clearly observed that both *M. verrucaria* and *G. roseum* enter the conidia of *A. brassicae* and grow inside the conidia. No specialized absorptive structures were seen, indicating that both *M. verrucaria* and *G. roseum* are necrotrophic mycoparasites.

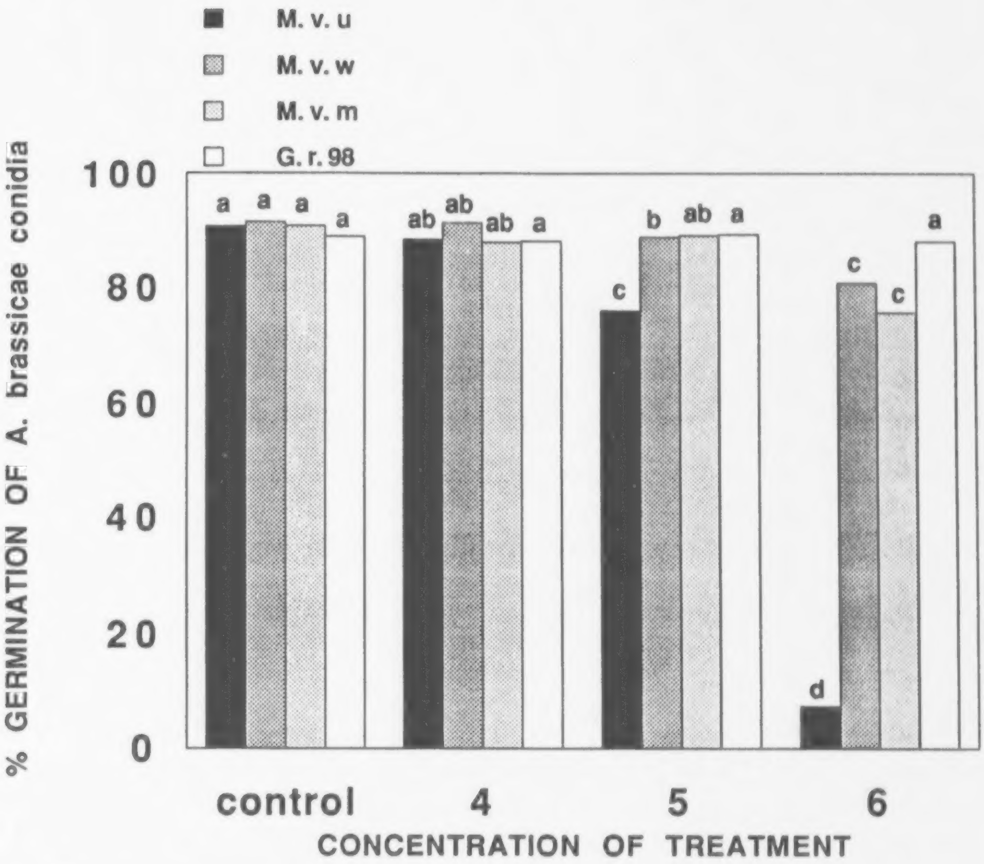
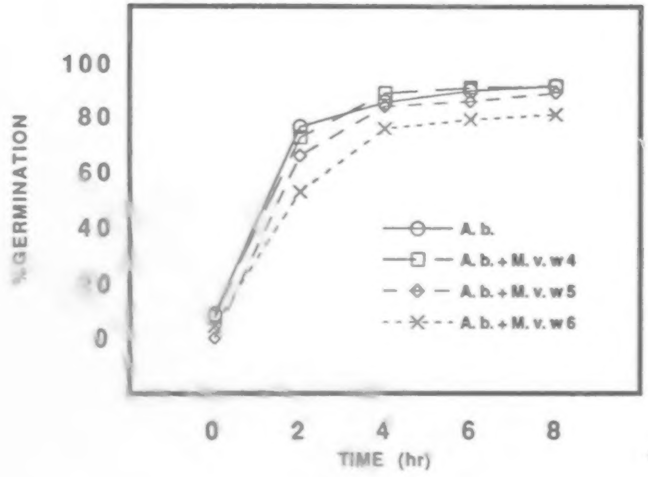


Figure 3.1. Comparison of the germination of *Alternaria brassicae* conidia with treatments of differing spore concentrations after 8 hours of incubation. Treatment concentrations are the number of spores/mL of the antagonist or equivalent amount of metabolites, with the *A. brassicae* control not being combined with *Gliocladium roseum* isolate 98, washed or unwashed conidia of *M. verrucaria* or metabolites of *M. verrucaria*. Comparisons were made within major treatment within time and are indicated by a, b, c, d. (M. v. = *M. verrucaria*, u = unwashed conidia, w = washed conidia, m = metabolites, G. r. 98 = *G. roseum* isolate 98)

Figure 3.2. *Alternaria brassicae* germination over time when combined with differing concentrations of washed *Myrothecium verrucaria* conidia (top left), unwashed *M. verrucaria* conidia (top right), metabolites of *M. verrucaria* (bottom left) and *Gliocladium roseum* isolate 98 (bottom right). Error bars are present but the error was so insignificant, the bars are not easily observed. Errors presented range from 0.096 to 2.82 (standard error of the ls mean) when alpha = 0.05. (A. b. = *A. brassicae*, M. v. = *M. verrucaria*, u = unwashed conidia, w = washed conidia, m = metabolites of *M. verrucaria*, G. r. 98 = *G. roseum* isolate 98, the number designations 4, 5, and 6 refer to the spore concentration of antagonists or the amount of metabolites of *M. verrucaria*)


%GERMINATION
100
80
60
40
20
0
0
2
4
6
8
TIME (hr)
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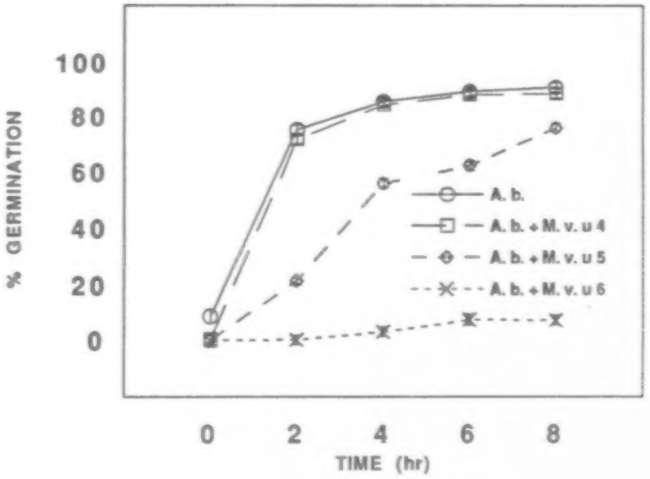
A. b. + M. v. w4
A. b. + M. v. w5
A. b. + M. v. w6


% GERMINATION
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A. b. + M. v. u4
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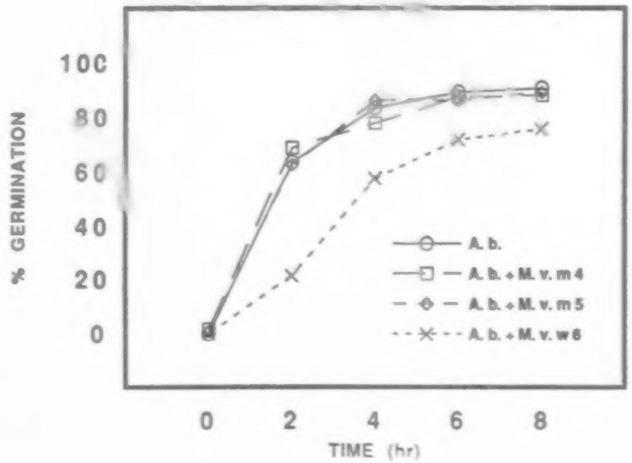
A. b. + M. v. u6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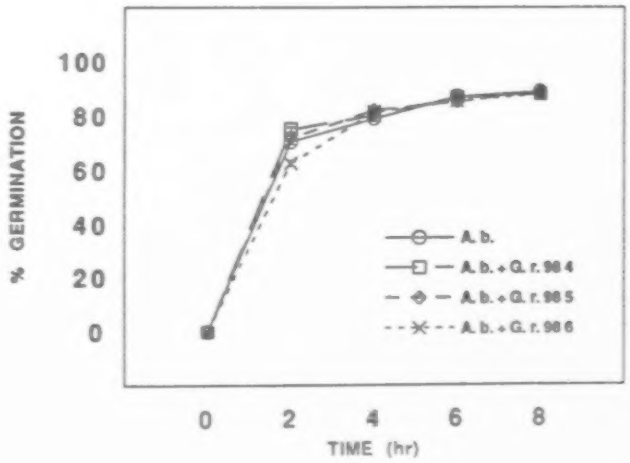
A. b. + M. v. m5
A. b. + M. v. w6


% GERMINATION
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80
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TIME (hr)
A. b.
A. b. + G. r. 984
A. b. + G. r. 985
A. b. + G. r. 986

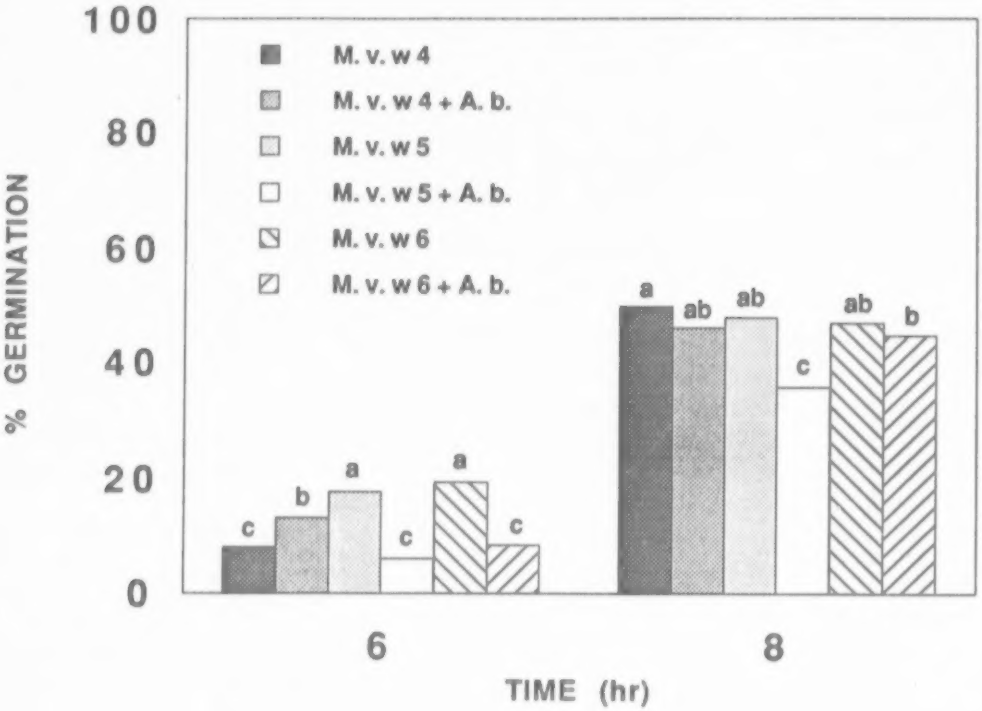


Figure 3.3. Germination of the washed conidia of *Myrothecium verrucaria* alone and in combination with *Alternaria brassicae*. Comparisons of percent germination within each time are indicated by a, b, c. (A. b. = *A. brassicae*, M. v. = *M. verrucaria*, w = washed conidia, the number designations 4, 5, and 6 refer to the spore concentration of washed conidia of *M. verrucaria*)

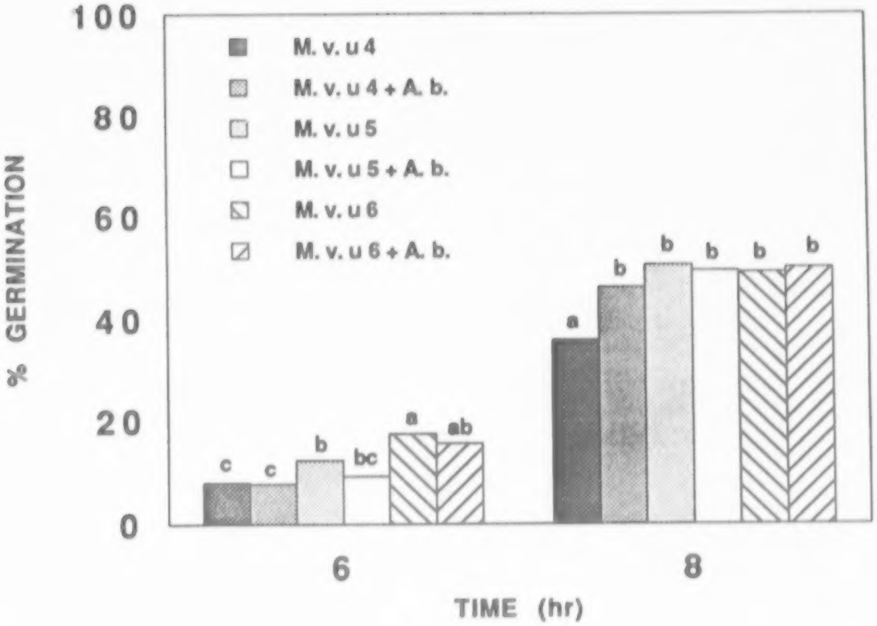


Figure 3.4. Germination of the unwashed conidia of *Myrothecium verrucaria* alone and in combination with *Alternaria brassicae*. Comparisons of percent germination within each time are indicated by a, b, c. (A. b. = *A. brassicae*, M. v. = *M. verrucaria*, u = unwashed conidia, the number designations 4, 5, and 6 refer to the spore concentration of unwashed conidia of *M. verrucaria*)

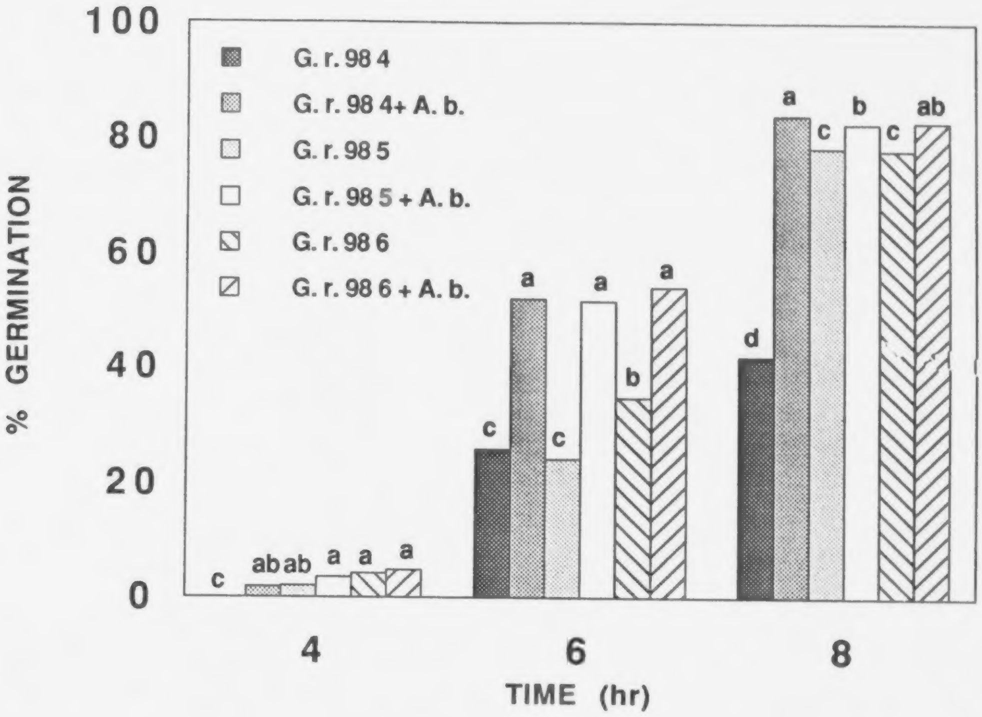


Figure 3.5. Germination of conidia of *Gliocladium roseum* isolate 98 alone and in combination with *Alternaria brassicae*. Comparisons of percent germination within each time are indicated by a, b, c, d. (A. b. = *A. brassicae*, G. r. 98 = *G. roseum* isolate 98, the number designations 4, 5, and 6 refer to the spore concentration of *G. roseum* isolate 98 conidia)

Figure 3.6. Germinated conidium of *Alternaria brassicae* after 8 hours (control). (X 600)

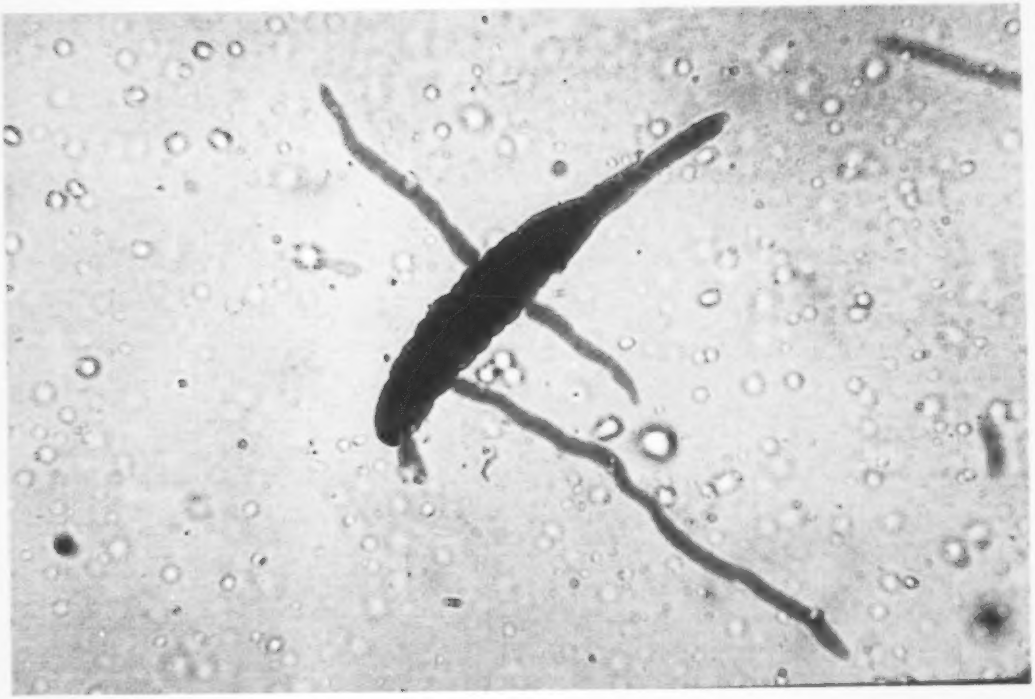
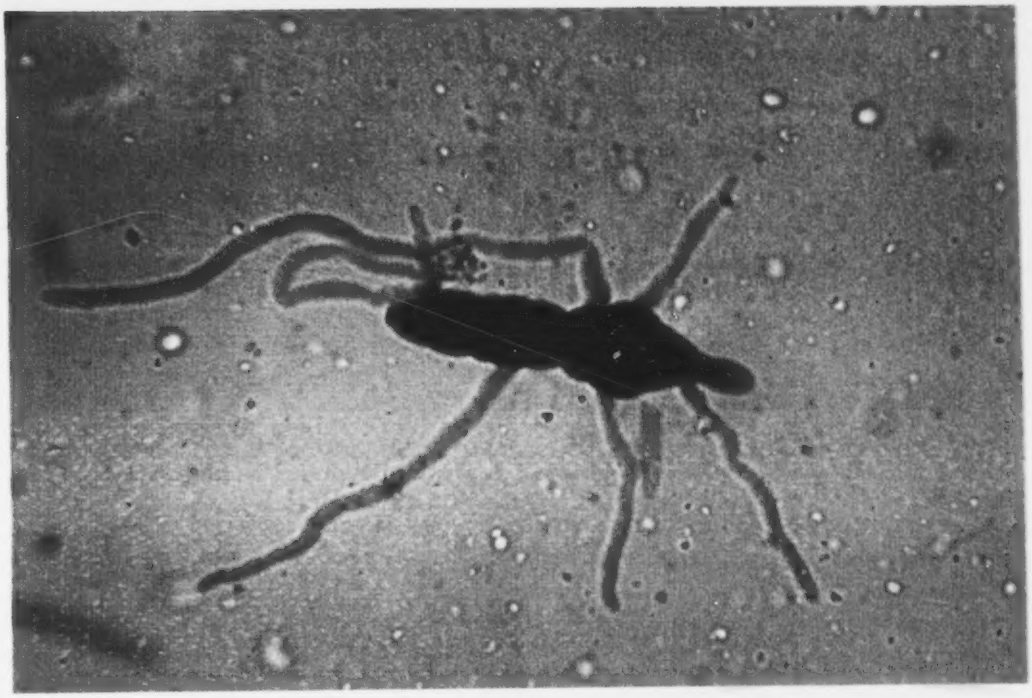


Figure 3.7. Germinated conidia of *Alternaria brassicae* in combination with *Myrothecium verrucaria* washed conidia (top) and unwashed conidia (bottom) at a concentration of 10^4 spores/mL after 8 hours. (X 600)



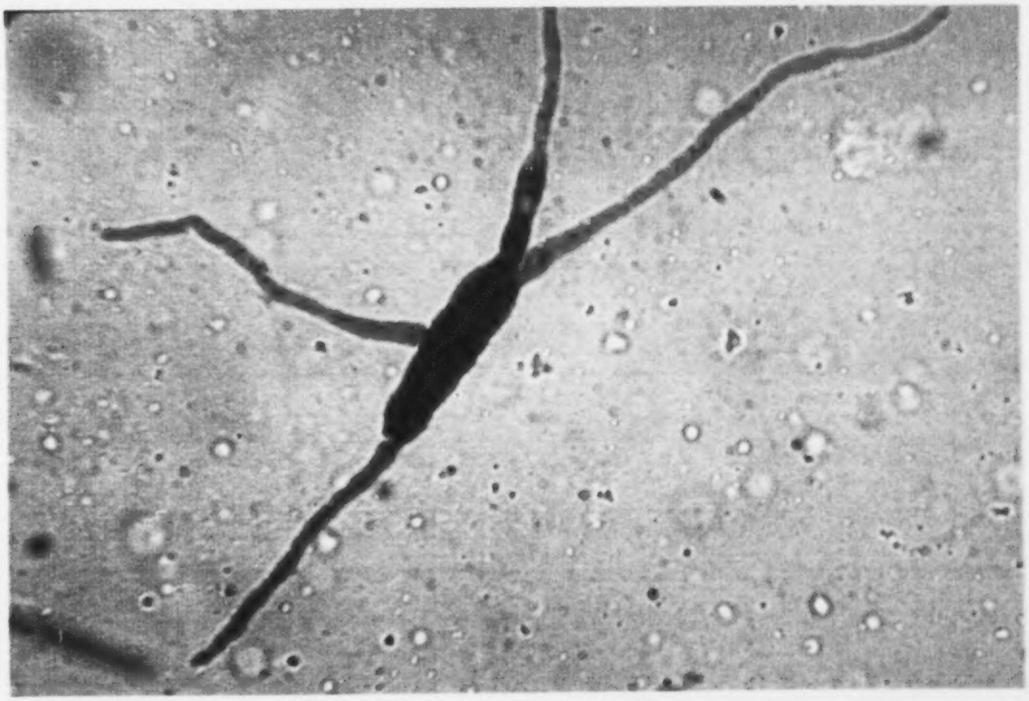
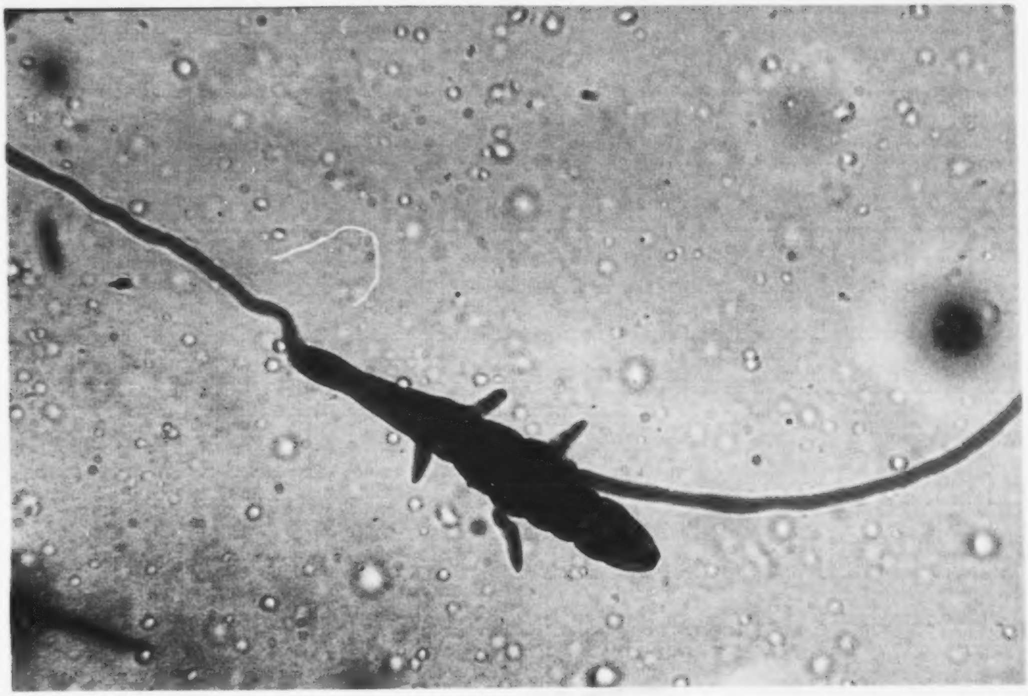


Figure 3.8. Germinated conidia of *Alternaria brassicae* in combination with metabolites of *Myrothecium verrucaria* (top) and conidia of *Gliocladium roseum* isolate 98 (bottom) at a concentration of 10^4 spores/mL after 8 hours. (X 600)



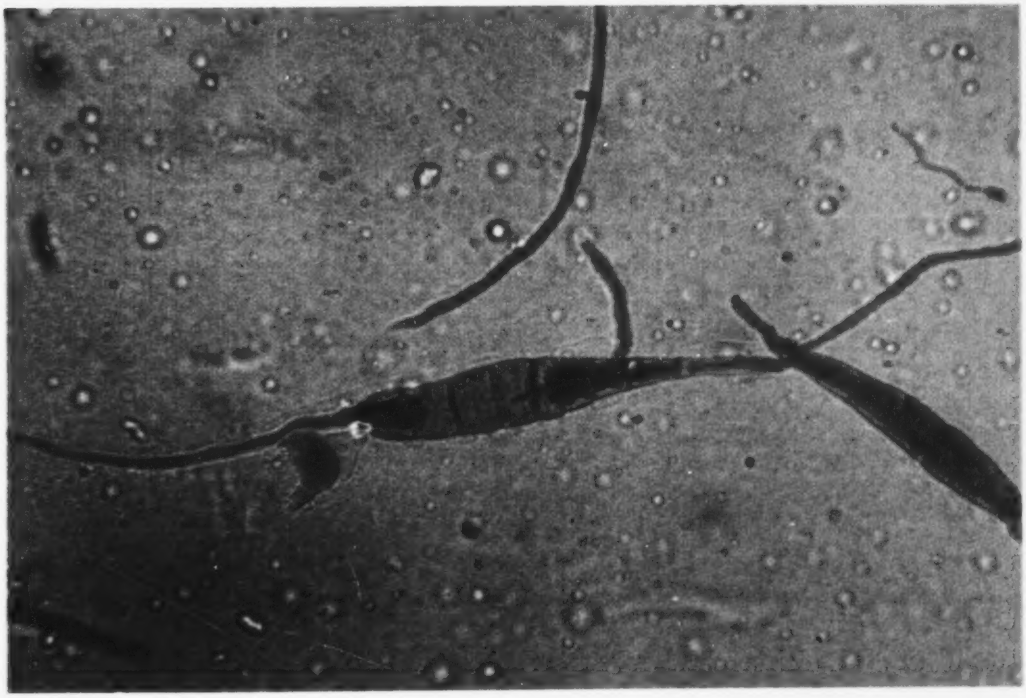
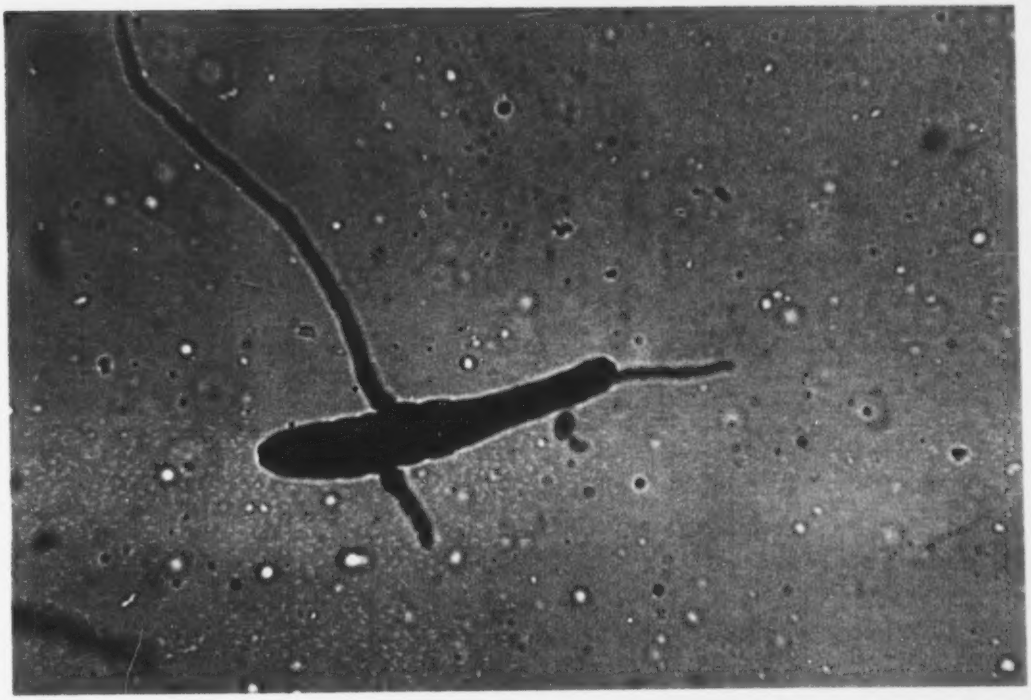


Figure 3.9. Germinated conidia of *Alternaria brassicae* in combination with *Myrothecium verrucaria* washed conidia (top) and unwashed conidia (bottom) at a concentration of 10^5 spores/mL after 8 hours. (X 600)



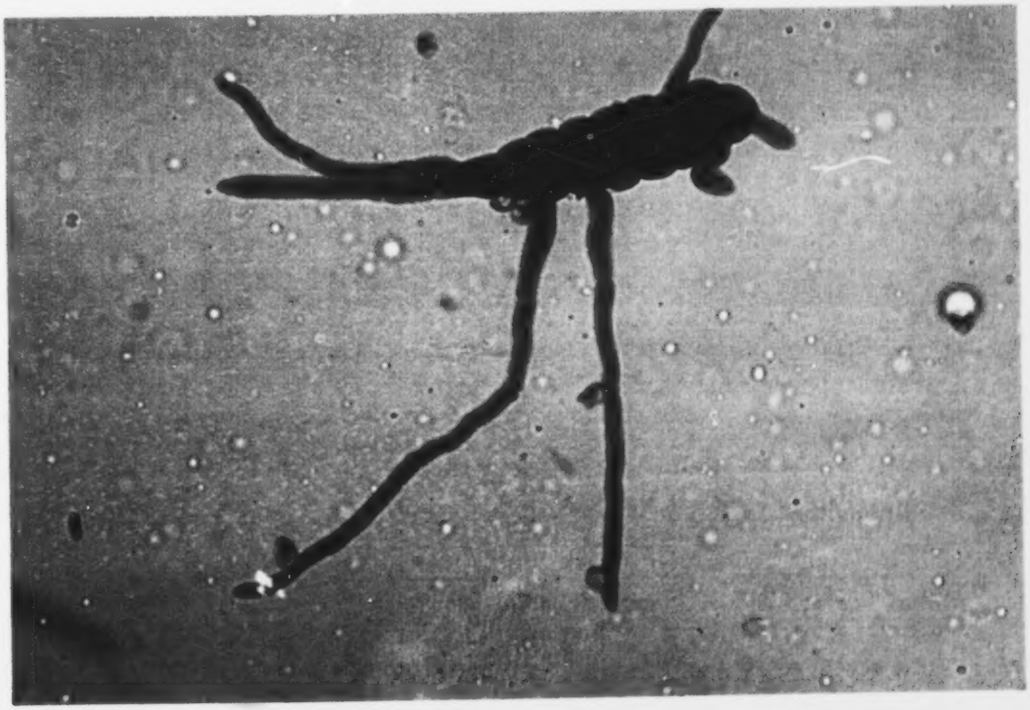
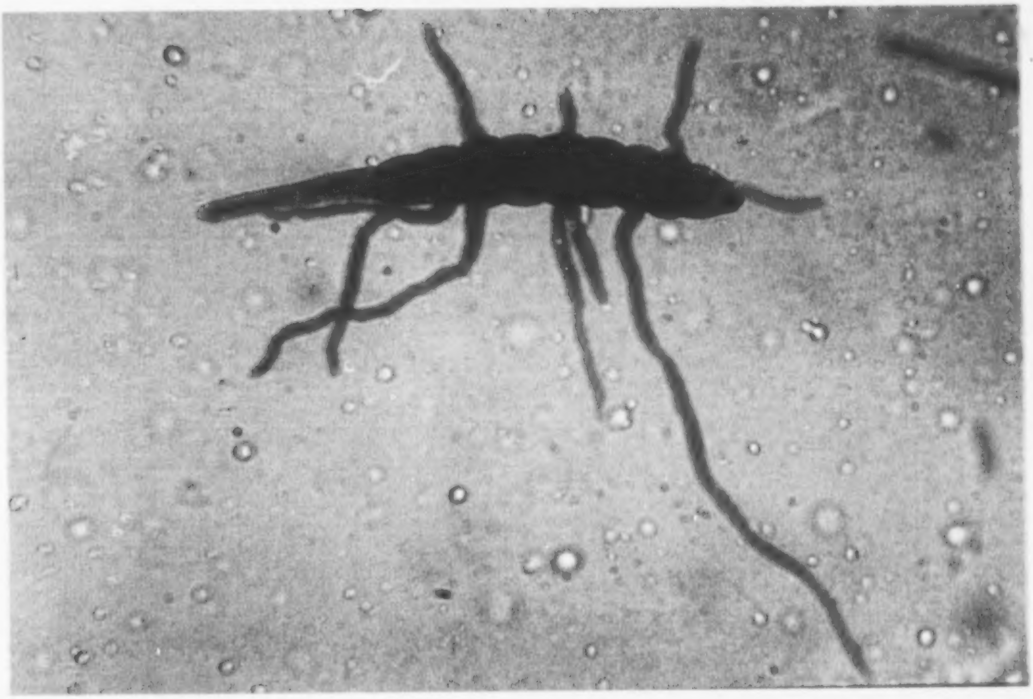


Figure 3.10. Germinated conidia of *Alternaria brassicae* in combination with metabolites of *Myrothecium verrucaria* (top) and *Gliocladium roseum* isolate 98 (bottom) at a concentration of 10^5 spores/mL after 8 hours. (X 600)



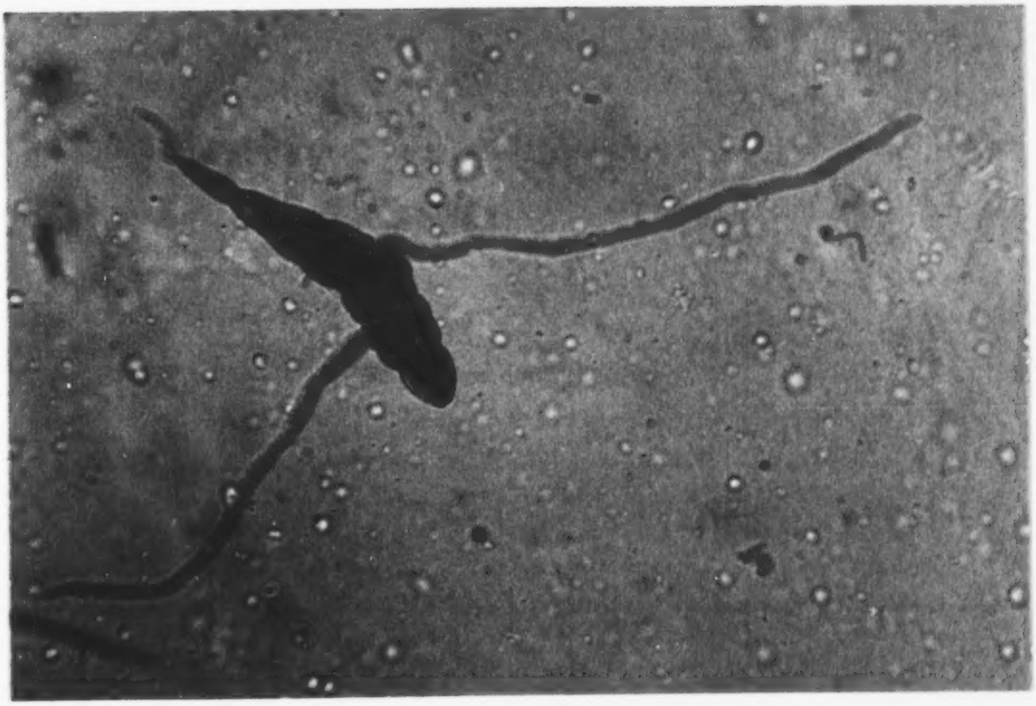
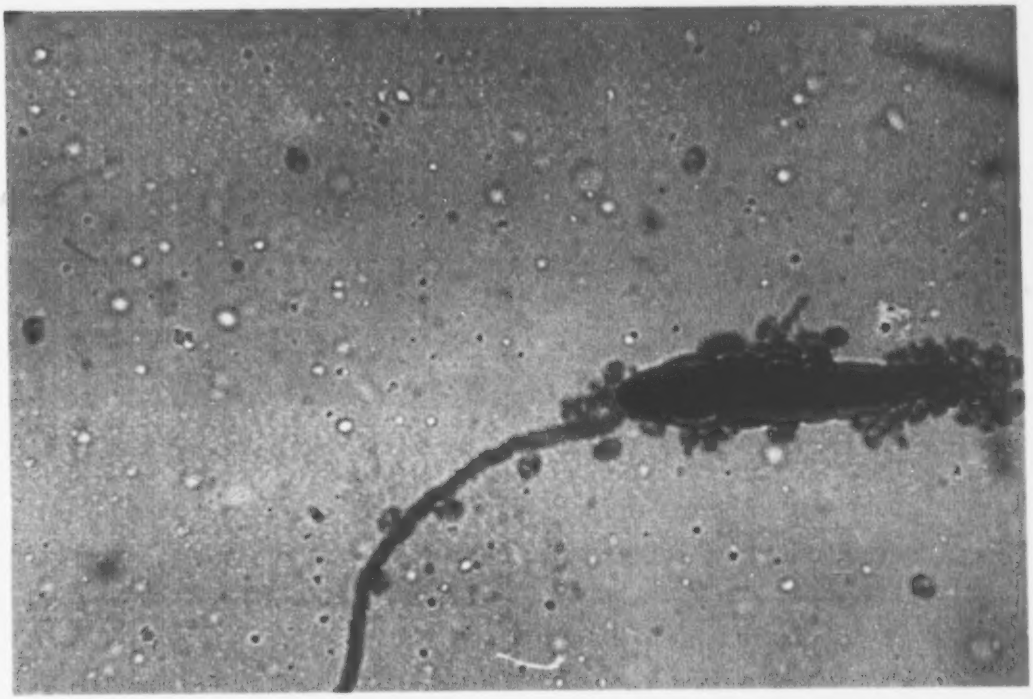


Figure 3.11. Germinated conidia of *Alternaria brassicae* in combination with washed conidia of *Myrothecium verrucaria* (top) and unwashed conidia of *M. verrucaria* (bottom) at a concentration of 10^6 spores/mL after 8 hours. Note that the germ tube lengths of *A. brassicae* conidia combined with washed *M. verrucaria* are slightly shorter than those of the control and that *A. brassicae* conidia combined with unwashed conidia of *M. verrucaria* either failed to germinate or produced significantly shorter germ tubes. (X 600)



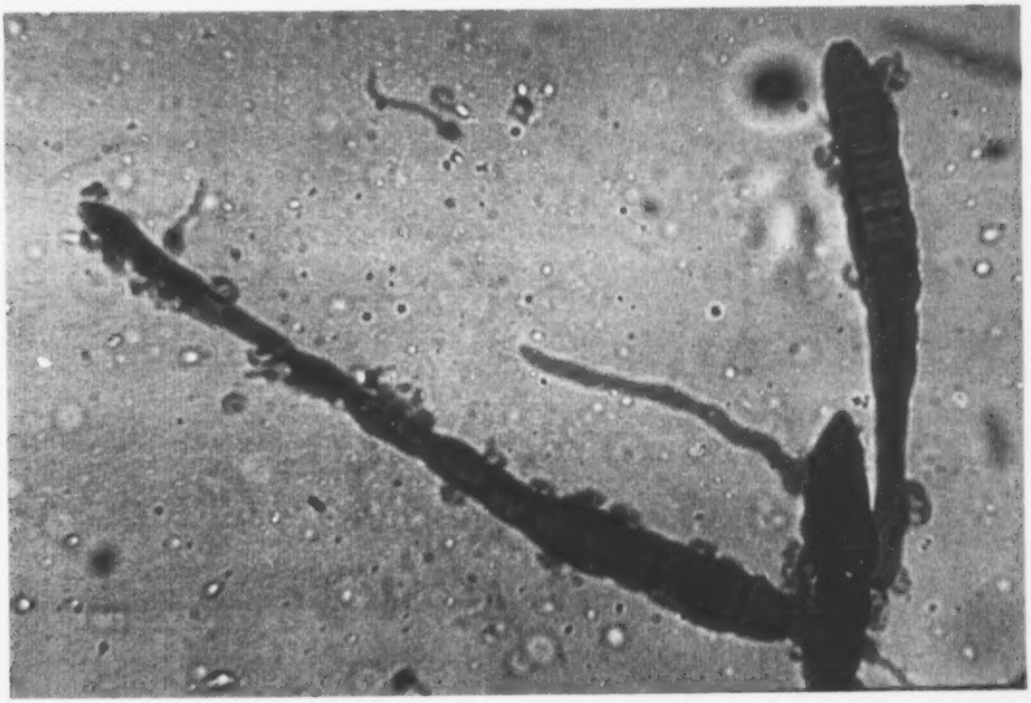
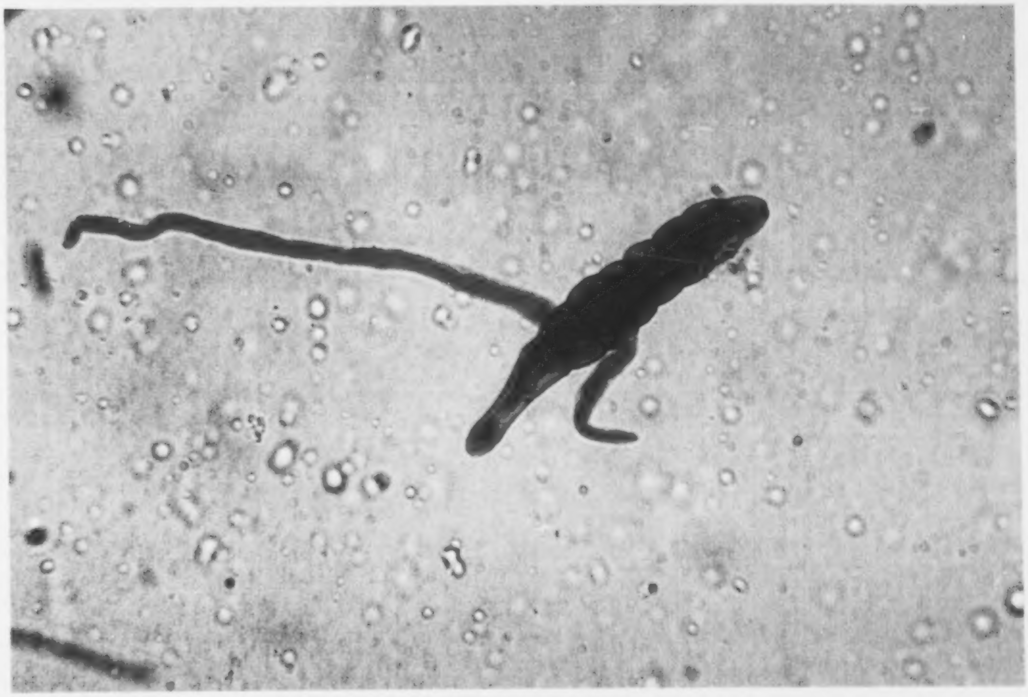


Figure 3.12. Germinated conidia of *Alternaria brassicae* in combination with metabolites of *Myrothecium verrucaria* (top) and *Gliocladium roseum* isolate 98 conidia (bottom) at a concentration of 10^6 spores/mL after 8 hours. (X 600)



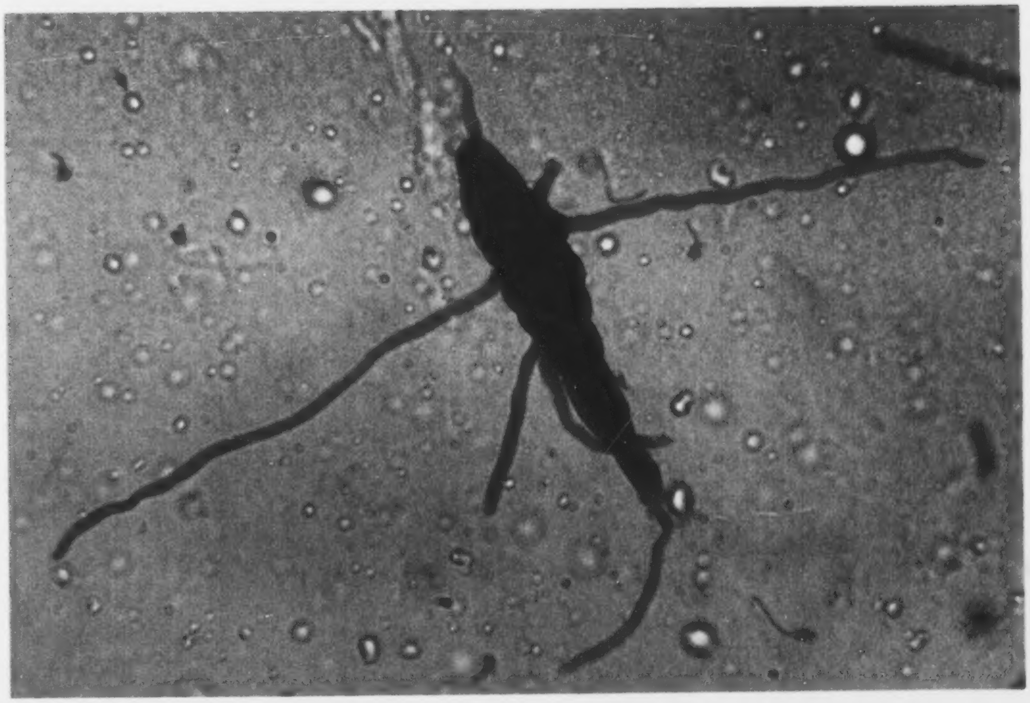


Figure 3.13. Inhibition of *Alternaria brassicae* at a distance when in co-culture with *Myrothecium verrucaria* 5 days after *M. verrucaria* was plated (top). Inhibition of *A. brassicae* and invasion of domain beginning when *A. brassicae* is in co-culture with *M.verrucaria* 10 days after *M. verrucaria* was plated (bottom). The right petri dish is an *A. brassicae* control plate. Invasion of domain occurs when the mycelium of one fungus partially or completely replaces the other.

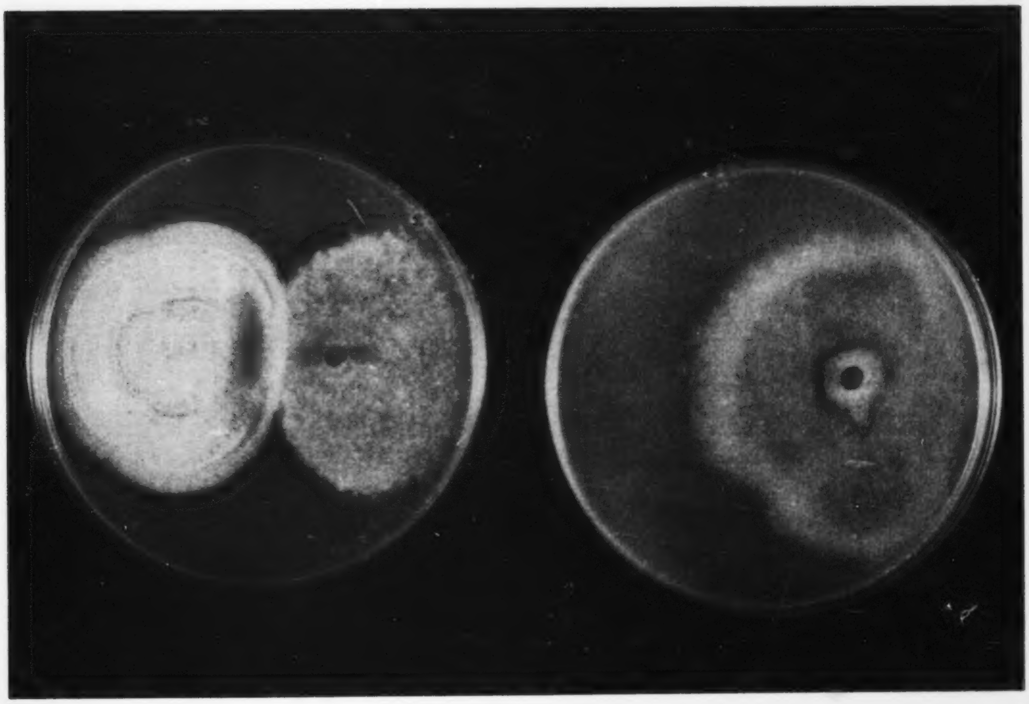
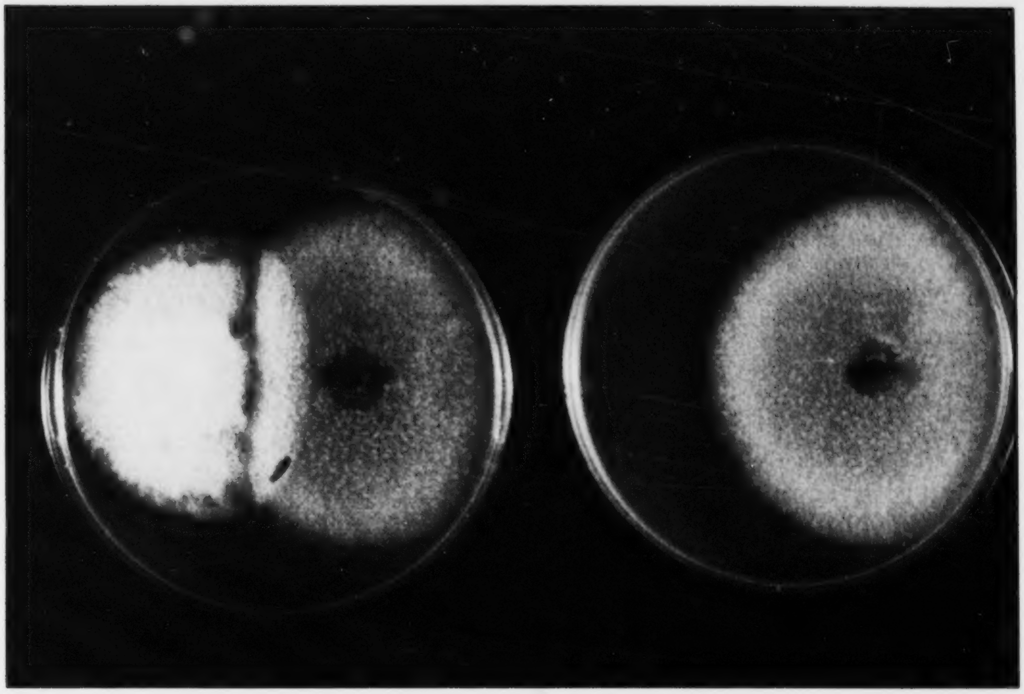


Figure 3.14. Invasion of domain and slight inhibition of *Alternaria brassicae* colonies by *Gliocladium roseum* isolate 98 (top) and *G. roseum* isolate 17 (bottom), 10 days after *G. roseum* was plated. The right petri dish is an *A. brassicae* control plate. Invasion of domain occurs when the mycelium of one fungus partially or completely replaces the other.



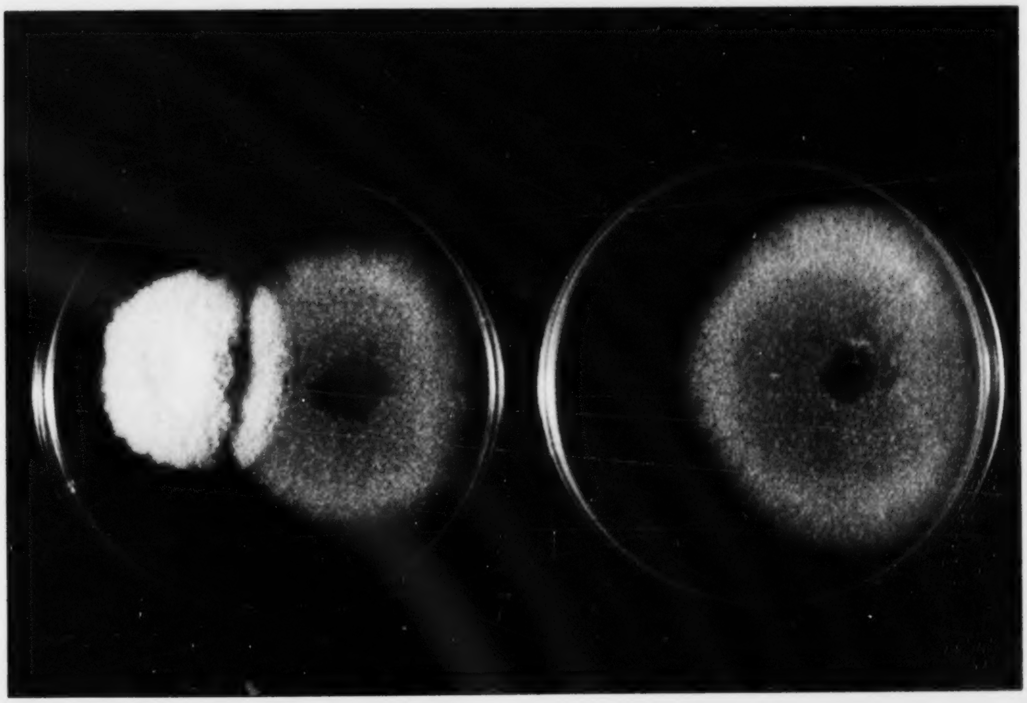


Figure 3.15. *Alternaria brassicae* control conidium and hyphae (top). Hyphae of *A. brassicae* parasitized by *Giocladium roseum* 15 days after *G. roseum* was plated (bottom). (X 600)

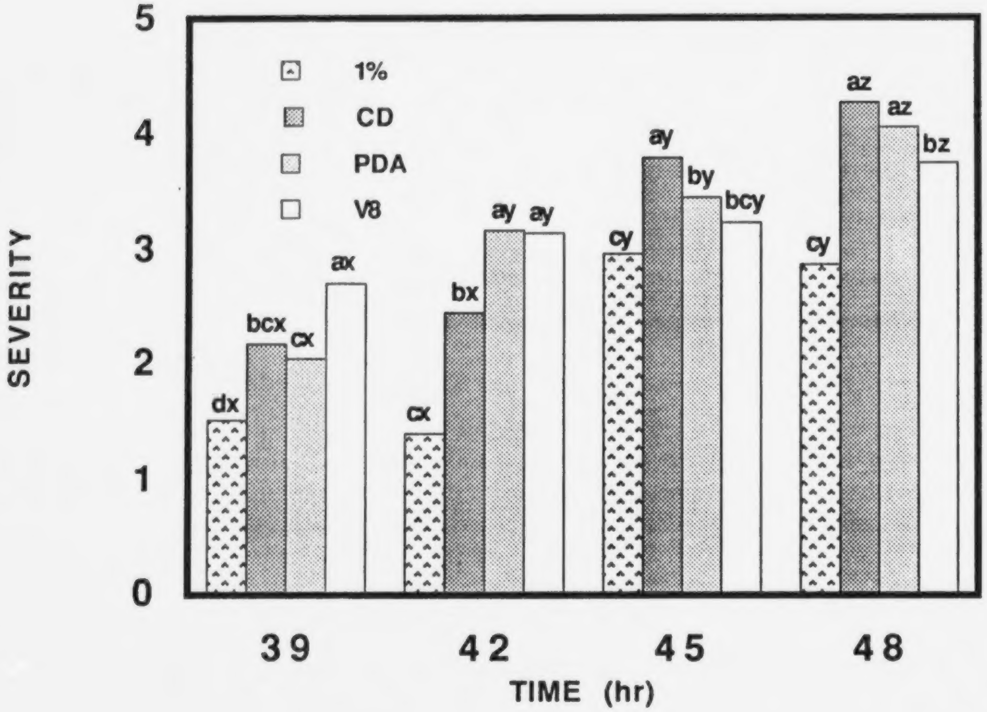


Figure 3.16. Severity of parasitism of *Alternaria brassicae* by *Gliocladium roseum* isolate 98 on different media. Comparisons of severity of parasitism occurring on the different media at the same time are noted with a, b, c, d. Comparisons of severity of parasitism occurring on the same media at different times are noted with x, y, z.

Figure 3.17. Parasitism of *Alternaria brassicae* by *Gliocladium roseum* isolate 98 on CD (top) and 1% water agar (bottom) after 48 hours. (X 600)

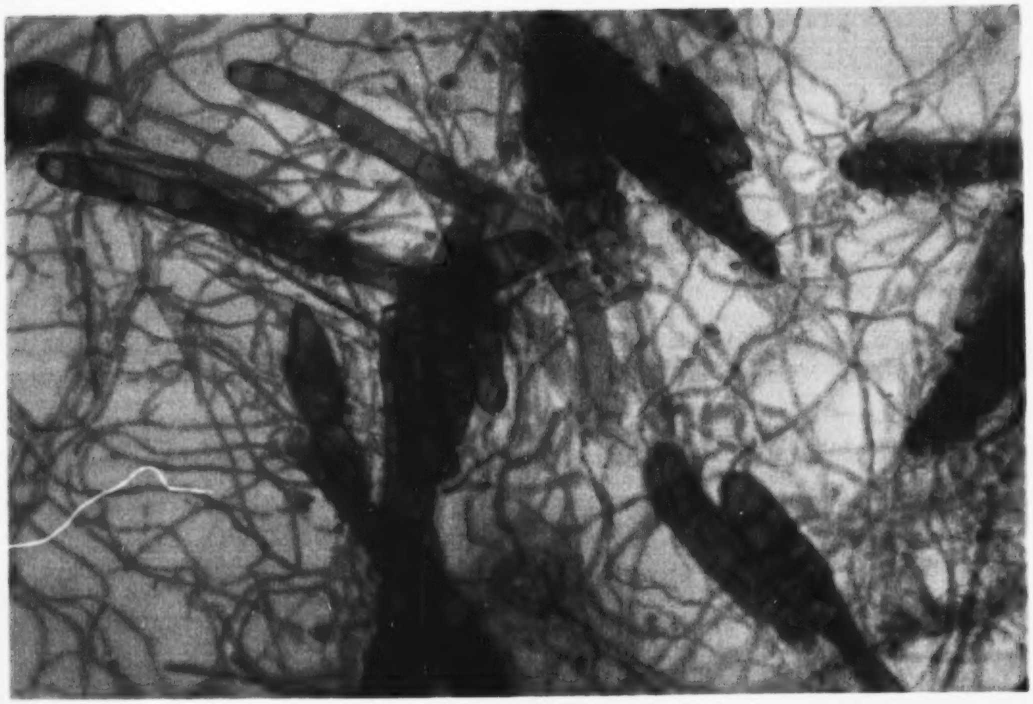
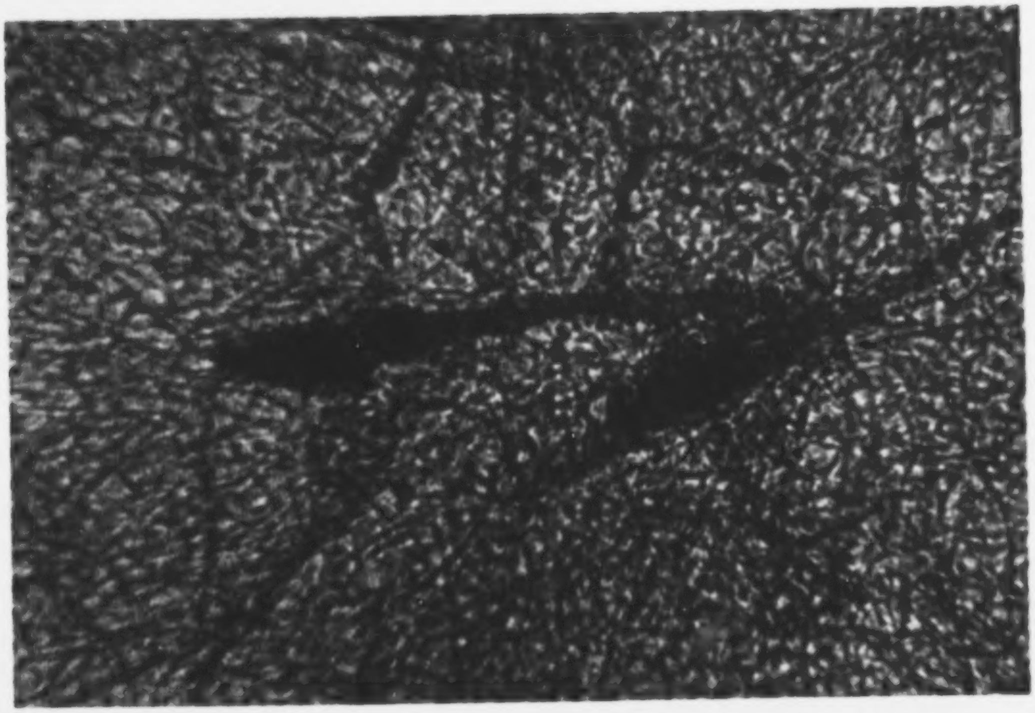


Figure 3.18. Parasitism of *Alternaria brassicae* by *Gliocladium roseum* isolate 98 on PDA (top) and V8 (bottom) after 48 hours. (X 600)



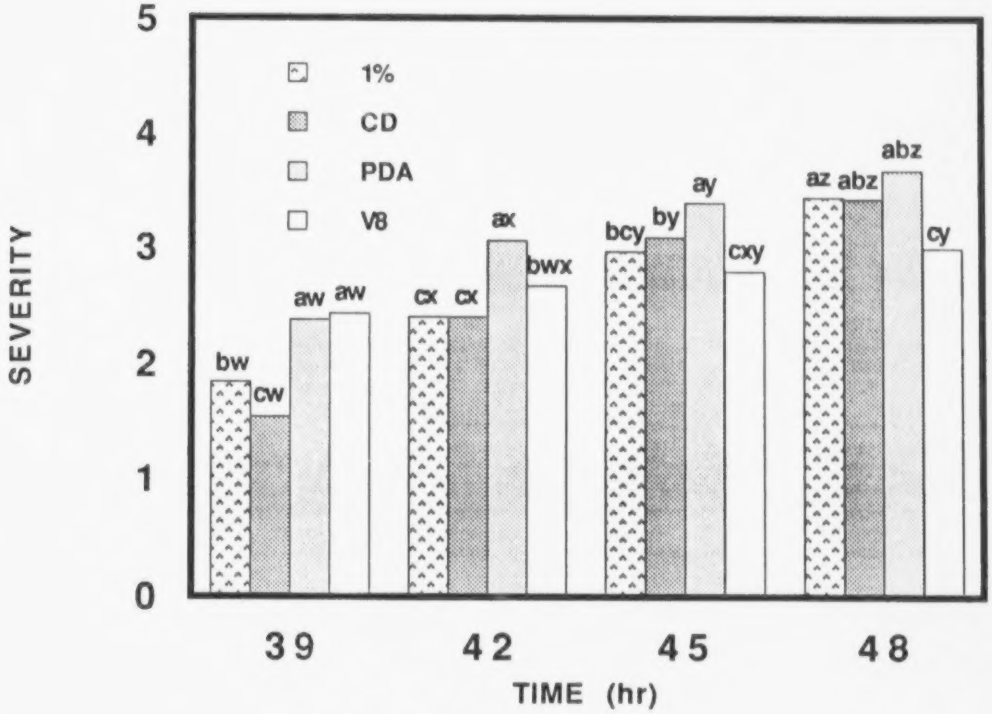


Figure 3.19. Severity of parasitism of *Alternaria brassicae* by *Myrothecium verrucaria* (grown from unwashed conidia) on different media. Comparisons of severity of parasitism occurring on the different media at the same time are noted with a, b, c. Comparisons of severity of parasitism occurring on the same media at different times are noted with w, x, y, z.

Figure 3.20. Parasitism of *Alternaria brassicae* by *Myrothecium verrucaria* (grown from unwashed conidia) on CD (top) and 1% water agar (bottom) after 48 hours. (X 600)

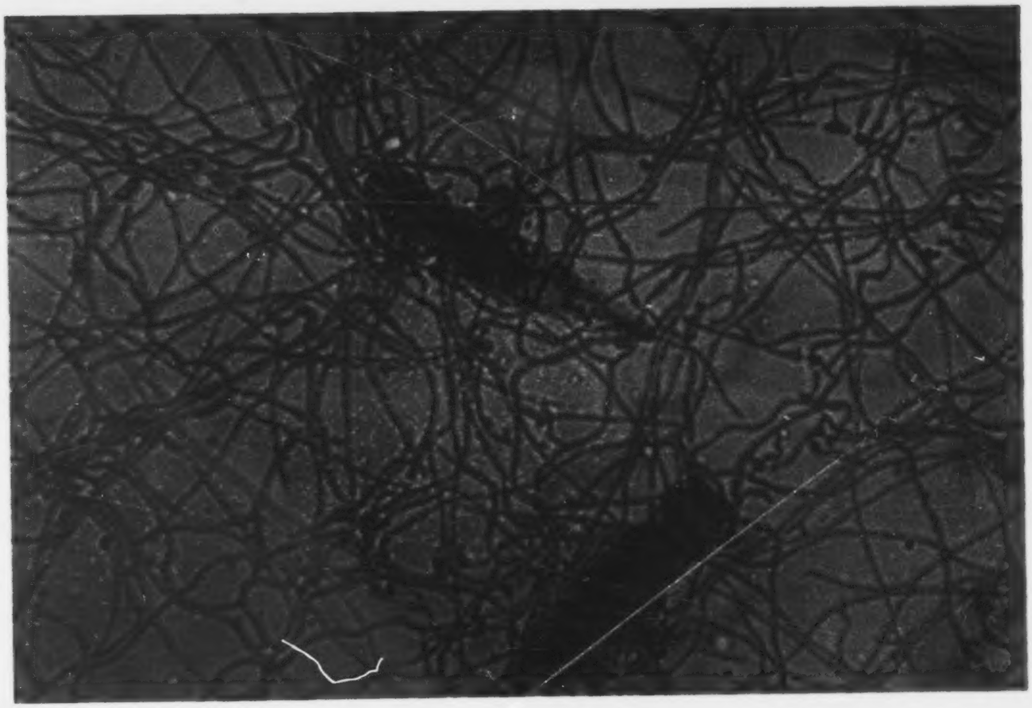
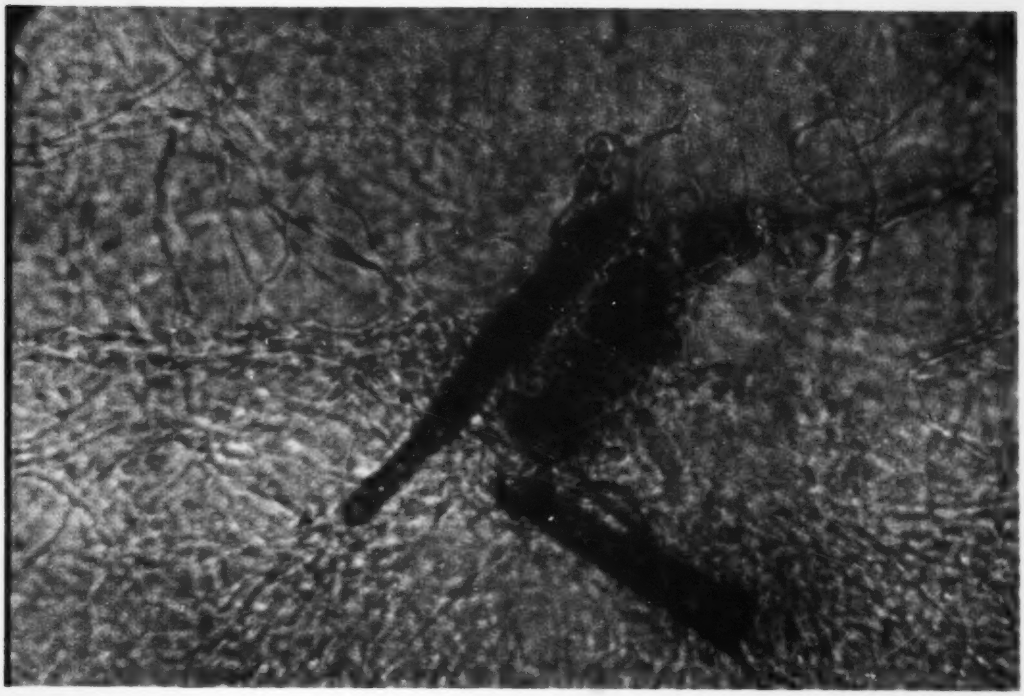


Figure 3.21. Parasitism of *Alternaria brassicae* by *Myrothecium verrucaria* (grown from unwashed conidia) on PDA (top) and V8 (bottom) after 48 hours. (X 600)



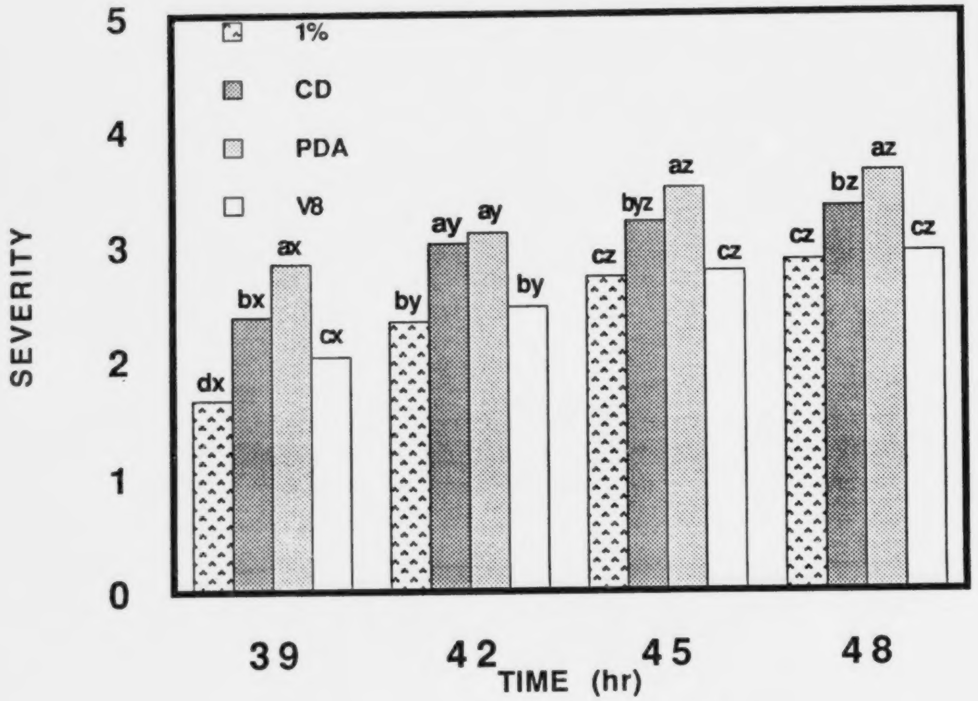


Figure 3.22. Severity of parasitism of *Alternaria brassicae* by *Myrothecium verrucaria* (grown from washed conidia) on different media. Comparisons of severity of parasitism occurring on the different media at the same time are noted with a, b, c, d. Comparisons of severity of parasitism occurring on the same media at different times are noted with x, y, z.

Figure 3.23. Parasitism of *Alternaria brassicae* by *Myrothecium verrucaria* (grown from washed conidia) on CD (top) and 1% water agar (bottom) after 48 hours. (X 600)

Figure 3.24. Parasitism of *Alternaria brassicae* by *Myrothecium verrucaria* (grown from washed conidia) on PDA (top) and V8 (bottom) after 48 hours. (X 600)

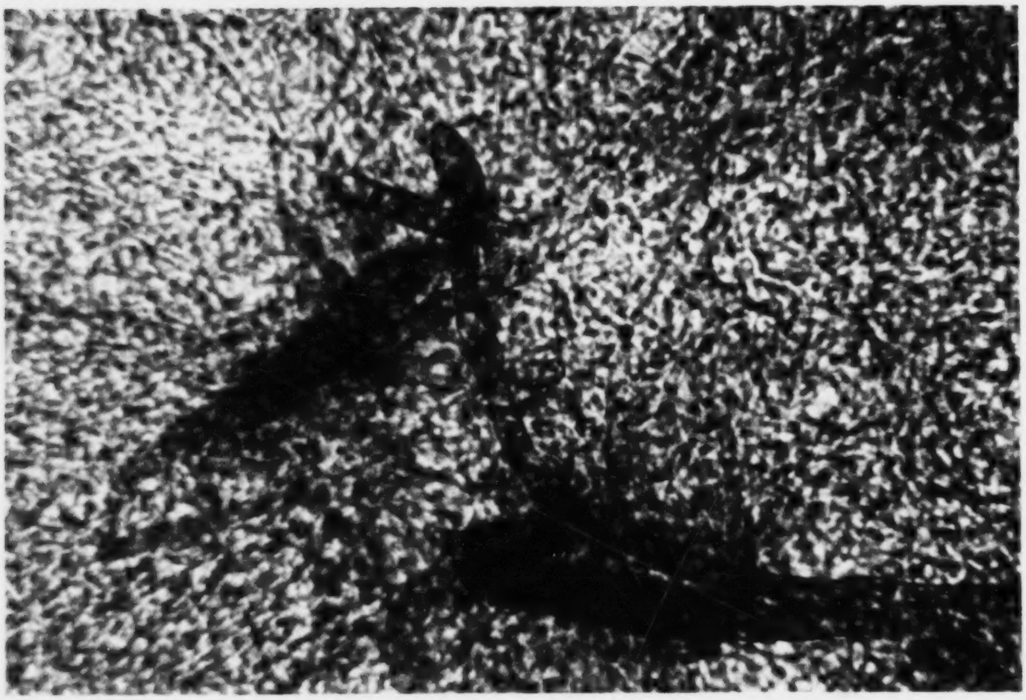


Table 3.1. Percentage of *Alternaria brassicae* conidia parasitized by *Myrothecium verrucaria* grown from washed and unwashed conidia and *Gliocladium roseum* isolate 98 grown from washed conidia on media of differing nutritional value

Time (hr) /media	39	42	45	48
1%	*72.5	73.4	94.2	94.2
	†69.2	85.0	91.7	95.0
	◊71.7	87.5	92.5	95.9
CD	*86.7	96.7	96.7	100
	†74.2	92.7	96.7	98.4
	◊60.0	84.2	97.5	99.2
PDA	*91.7	98.4	100	100
	†93.3	94.2	100	100
	◊85.9	96.7	100	100
V8	*95.8	100	100	100
	†80.0	88.4	97.5	98.4
	◊90.0	95.9	99.2	98.4

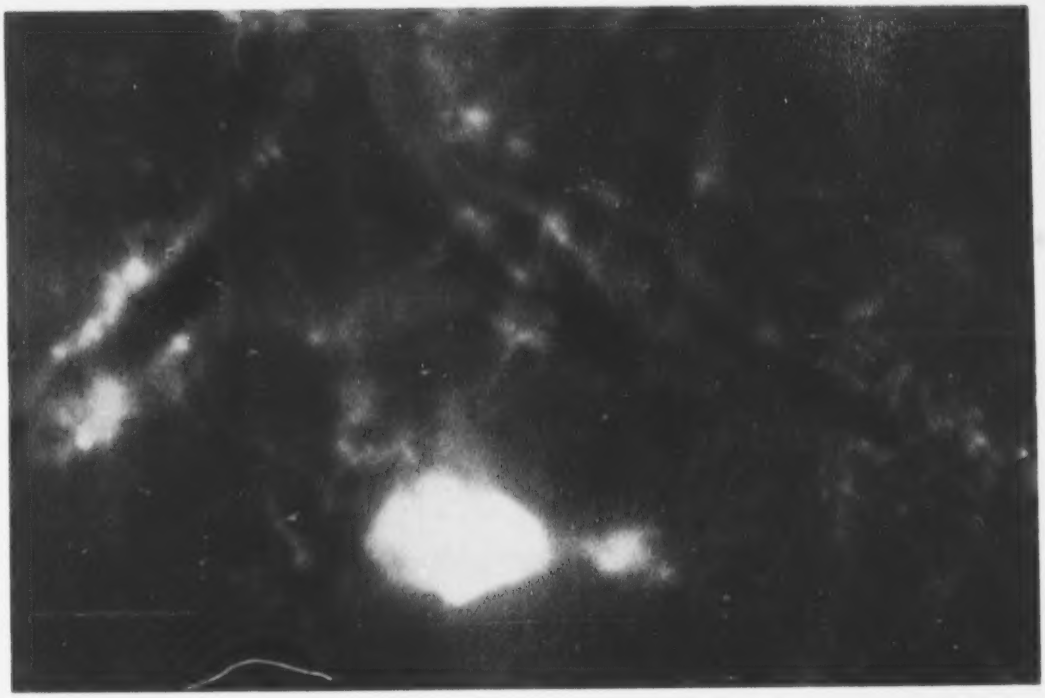
* indicates data for *G. roseum* isolate 98.

† indicates data for *M. verrucaria* grown from washed conidia.

◊ indicates data for *M. verrucaria* grown from unwashed conidia.

Figure 3.25. Hyphae and a conidium of *Alternaria brassicae* showing green fluorescence of hyphae and germinated cells after 48 hours of growth (top). Orange fluorescence of *A. brassicae* hyphae, a few cells of an older conidium and an entire young conidium is seen. The rest of the older conidium shows green fluorescence after 48 hours of growth (bottom). (X 600)

Figure 3.26. Orange fluorescence of the hyphae of *Myrothecium verrucaria* (grown from washed conidia) coiling around *Alternaria brassicae* conidia and some orange fluorescence of attacked cells of *A. brassicae* conidia after 48 hours (top). The tungsten light micrograph shows the same view (bottom). (X 600)



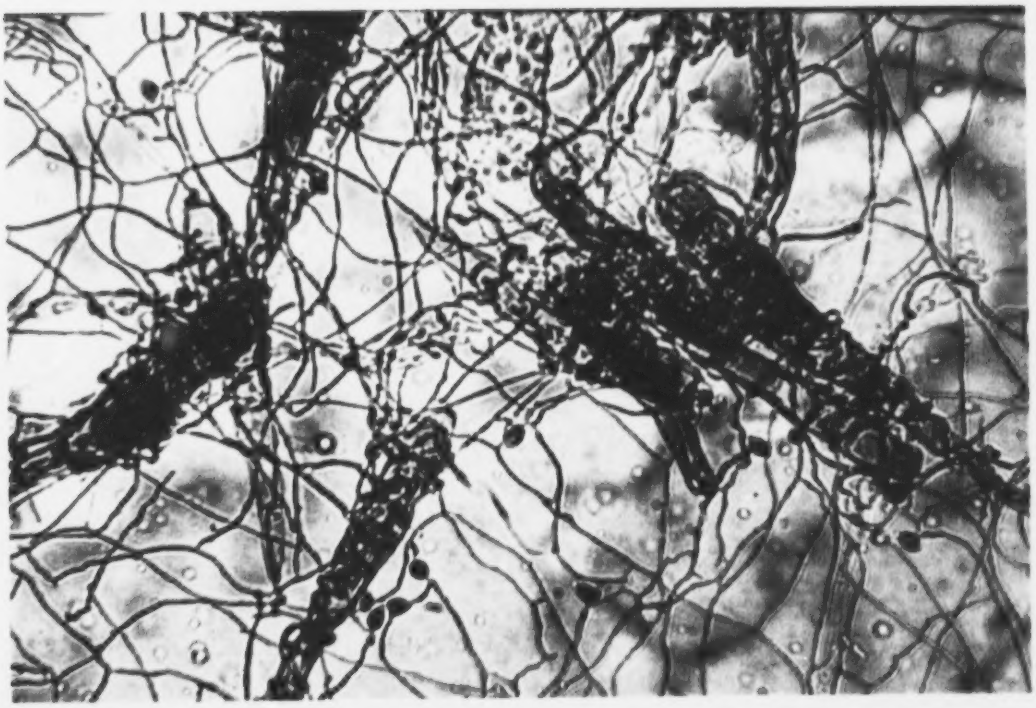


Figure 3.27. Conidia of *Alternaria brassicae* fluorescing green or orange when not in association or in association with *Myrothecium verrucaria,* respectively, after 39 hours (top). The hyphae of *A. brassicae* associated with *M. verrucaria* fluoresce orange (top). The associated tungsten light micrograph shows the same view (bottom). (X 600)

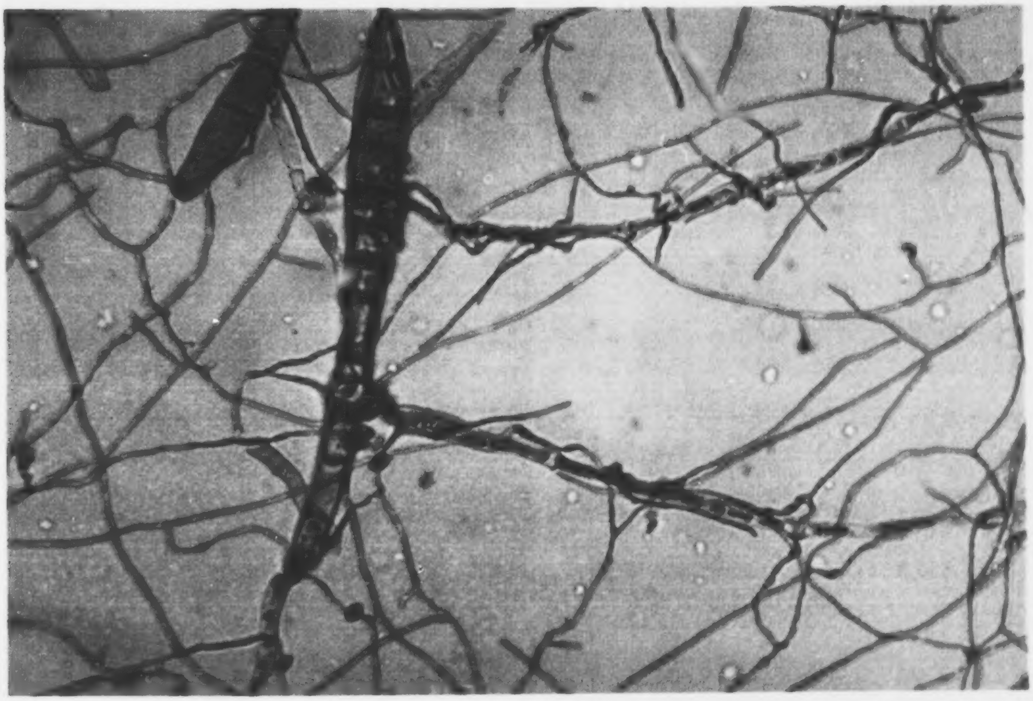


Figure 3.28. Orange fluorescence of *Alternaria brassicae* cells in association with *Myrothecium verrucaria* after 48 hours (top). The tungsten light micrograph shows the same view (bottom). Note that the cells exhibiting orange fluorescence are heavily parasitized by *M. verrucaria*. (X 600)

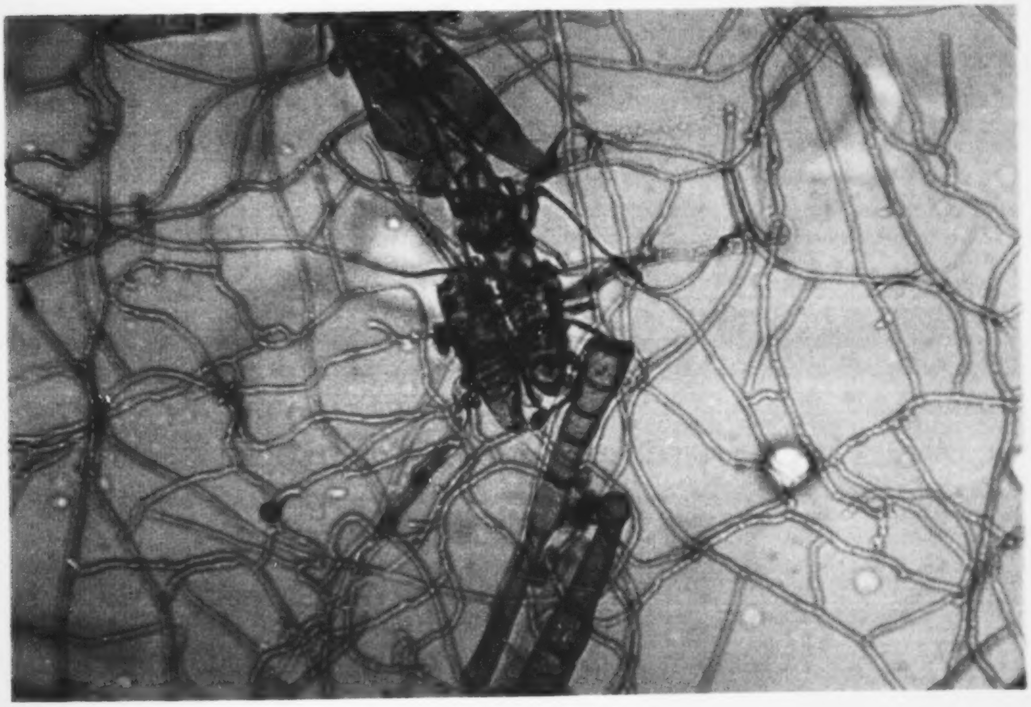


Figure 3.29. Orange fluorescence of *Alternaria brassicae* hyphae after 48 hours (top). The tungsten light micrograph shows the same view (bottom). Note that the hyphae in the tungsten light micrograph appear viable. (X 600)

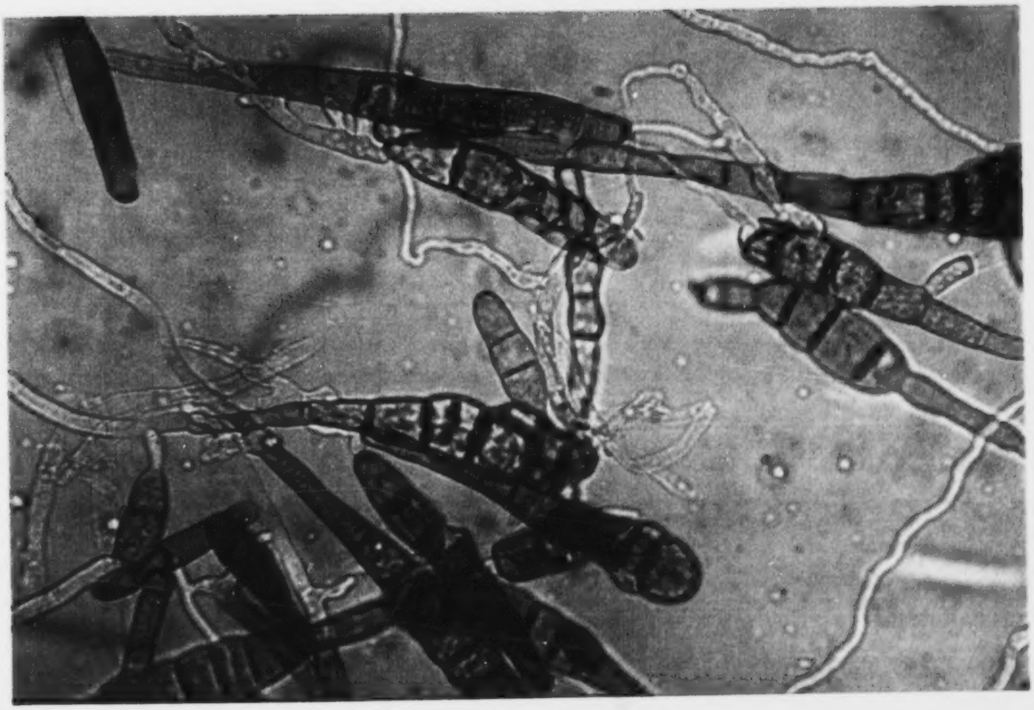
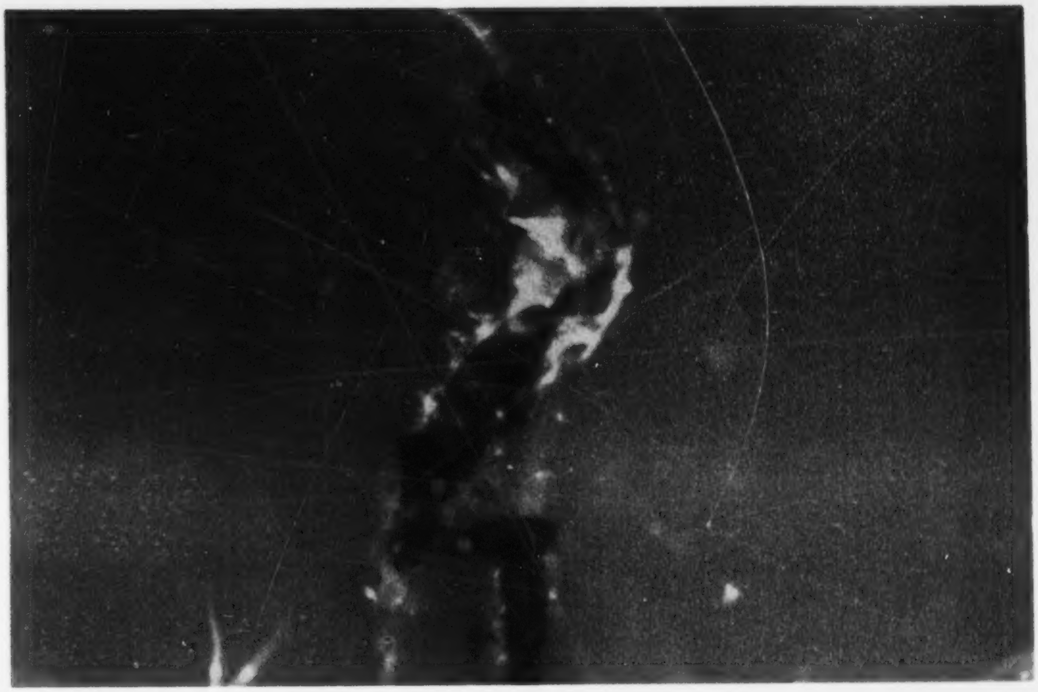


Figure 3.30. Orange fluorescence of *Gliocladium roseum* hyphae associated with *Alternaria brassicae* conidia after 48 hours (top). The tungsten light micrograph shows the same view (bottom). (X 600)



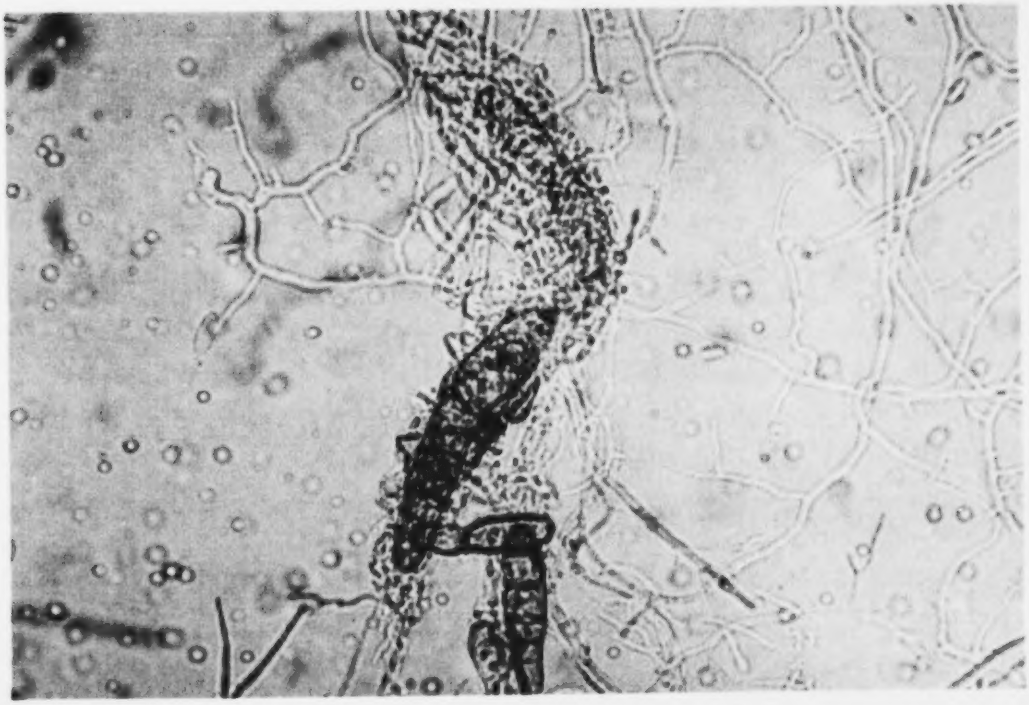
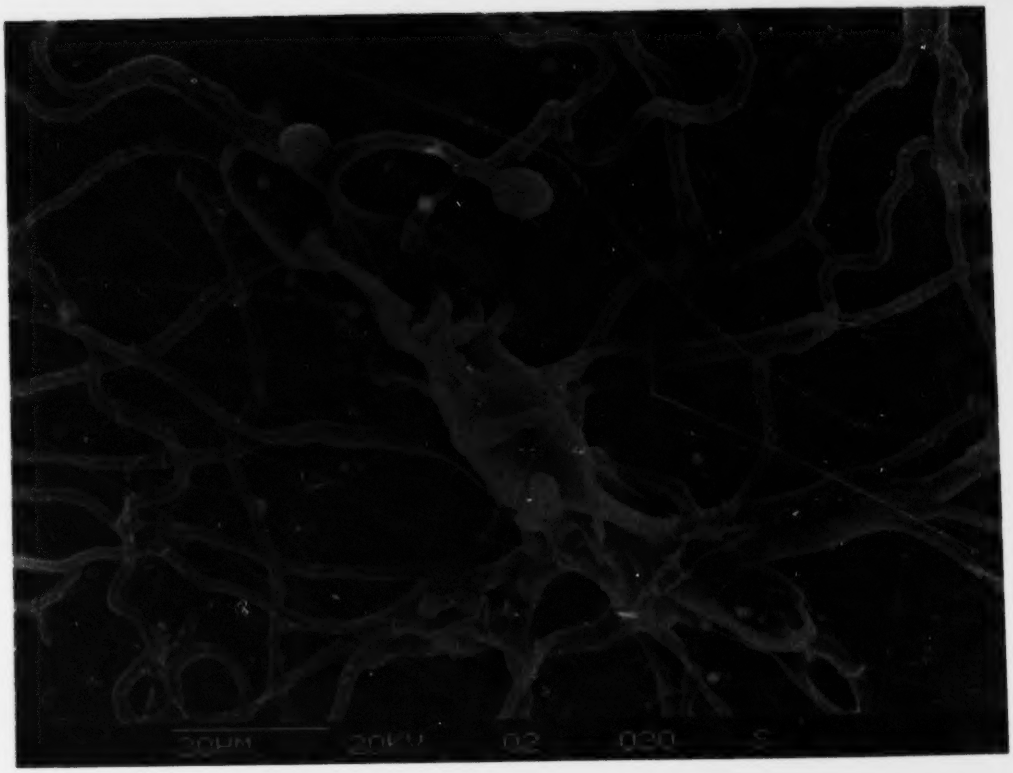


Figure 3.31. Scanning electron micrograph of *Myrothecium verrucaria* coiling around a conidium of *Alternaria brassicae* after 48 hours (top). Magnified view (bottom).


20µM
20KV
02
030
S

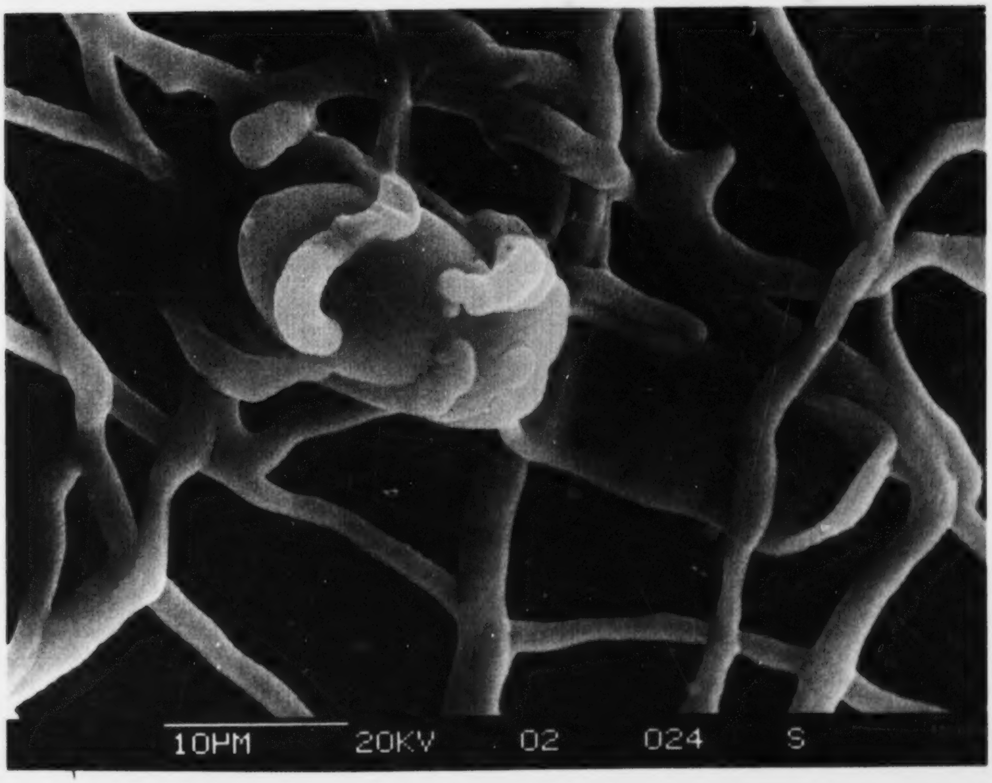
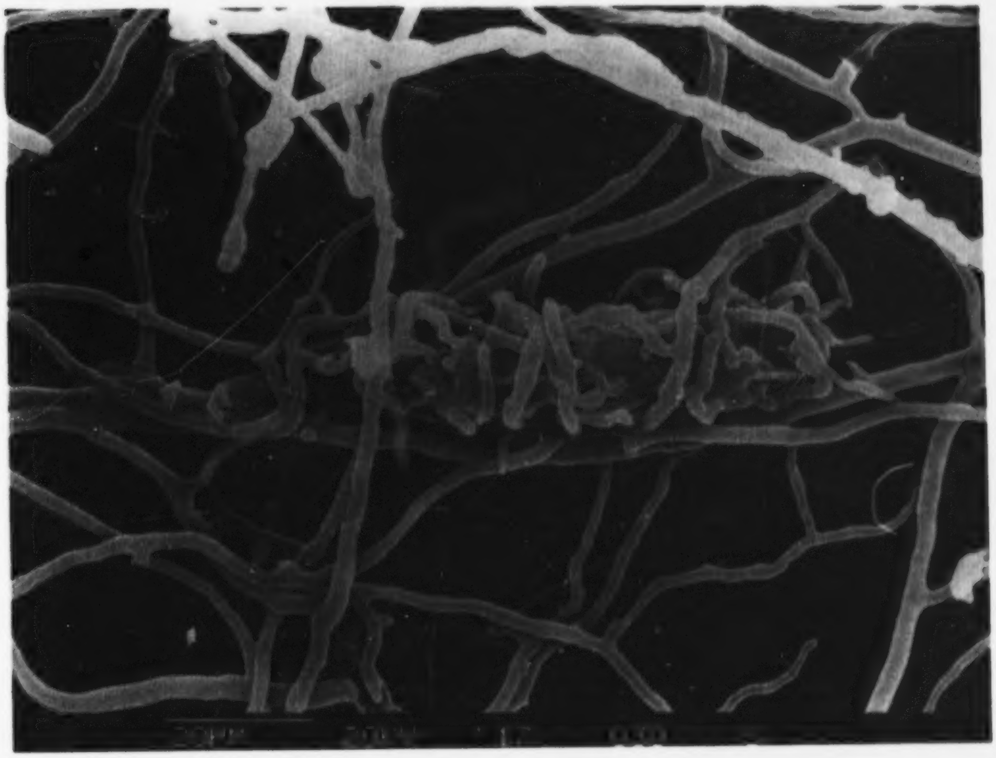

10µM
20KV
02
024
S

Figure 3.32. Scanning electron micrograph of *Gliocladium roseum* hyphae coiling around a conidium of *Alternaria brassicae* after 48 hours (top). Magnified view of *G. roseum* hyphae coiling around an *A. brassicae* conidium after 48 hours. A few short branches appear to be pre-penetration structures (bottom).



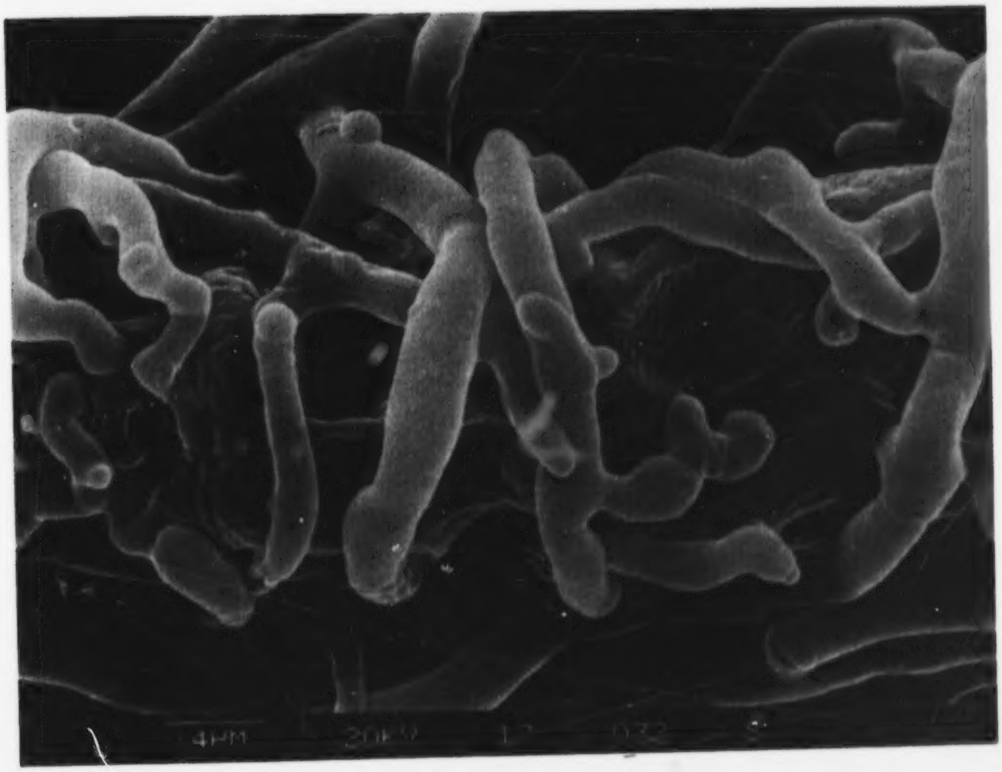
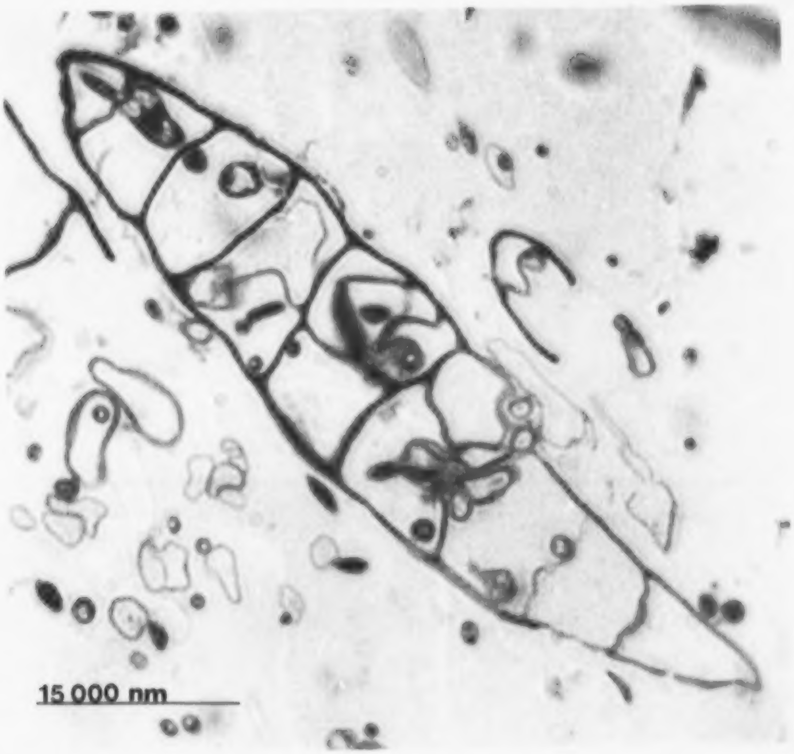

4µm
20KV

Figure 3.33. A thick section of *Myrothecium verrucaria* hyphae within an *Alternaria brassicae* conidium after 48 hours as viewed through the oil immersion lens of a light microscope (top) (X 1900). A thick section of a cross section of a conidium of *A. brassicae* being penetrated by *M. verrucaria* after 48 hours (bottom) (X 2000).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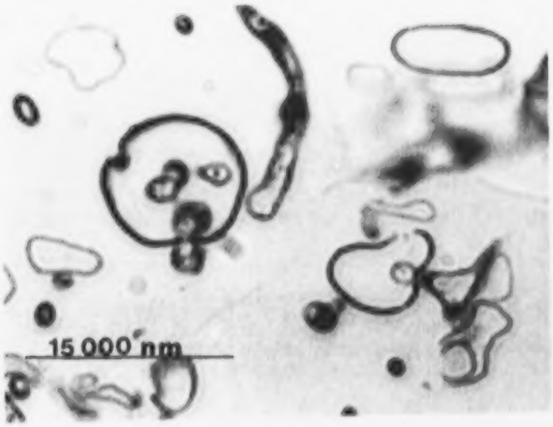

15 000 nm

Figure 3.34. A transmission electron micrograph of an ultrathin section showing *Myrothecium verrucaria* penetrating *Alternaria brassicae* after 48 hours. Note that the cell wall of *A. brassicae* is pushed inwards (top) (X 23 500). A transmission electron micrograph of an ultrathin section of *Alternaria brassicae* being penetrated on either side by *Myrothecium verrucaria* after 48 hours. Note the indentation and partial degradation of the *A. brassicae* cell wall (bottom) (X 13 500).

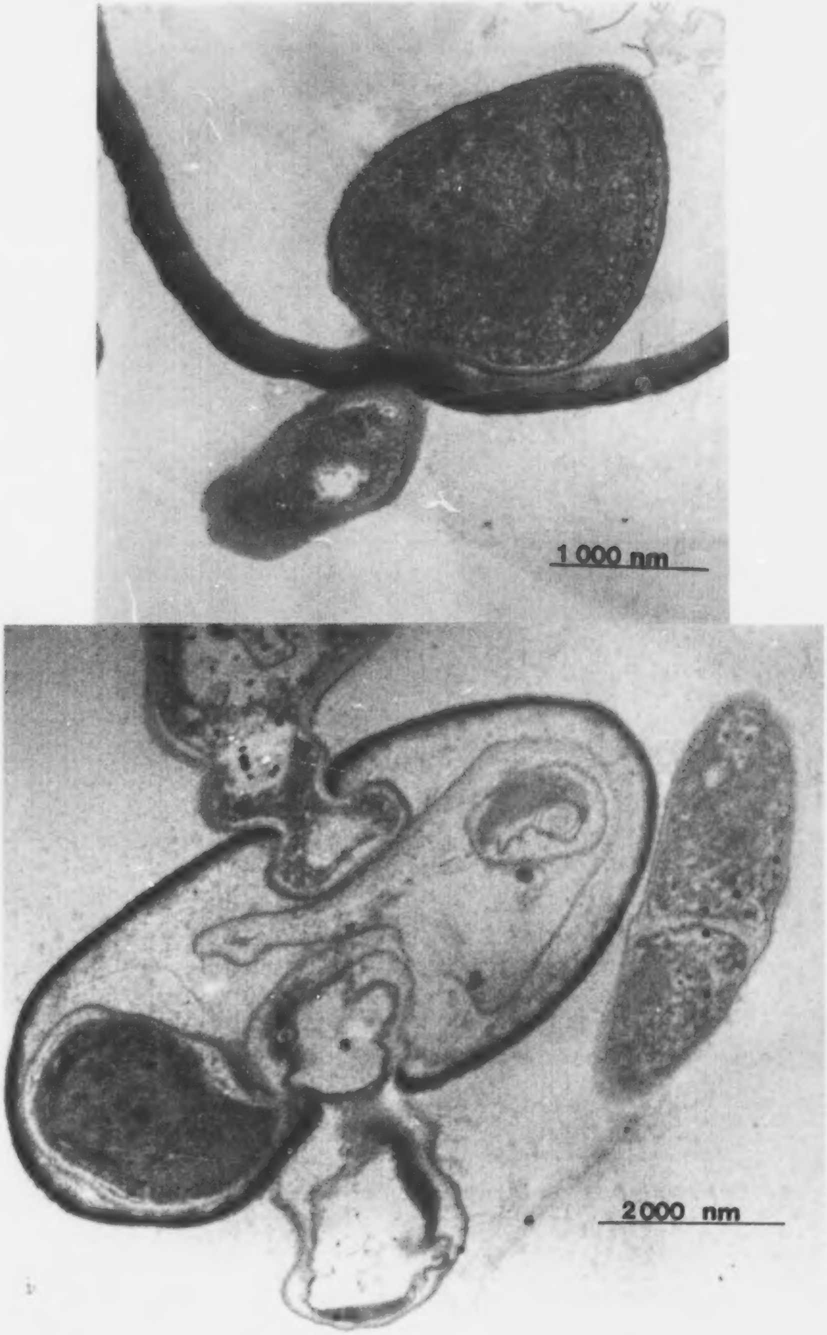
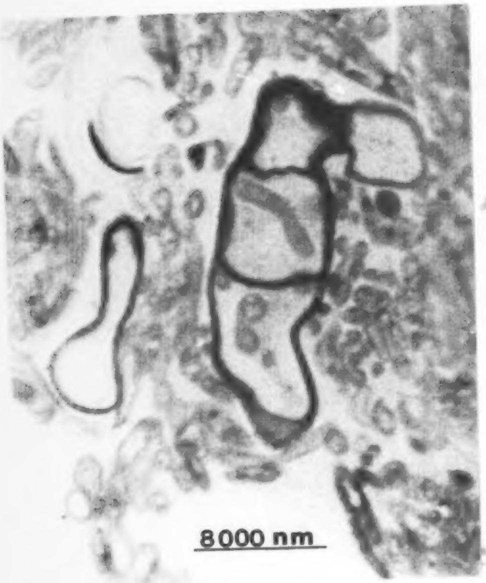

1000 nm
2000 nm

Figure 3.35. Light micrographs (as viewed through the oil immersion lens) of thick sections of *Gliocladium roseum* hyphae within conidia of *Alternaria brassicae* after 48 hours (X 2400).


8000 nm

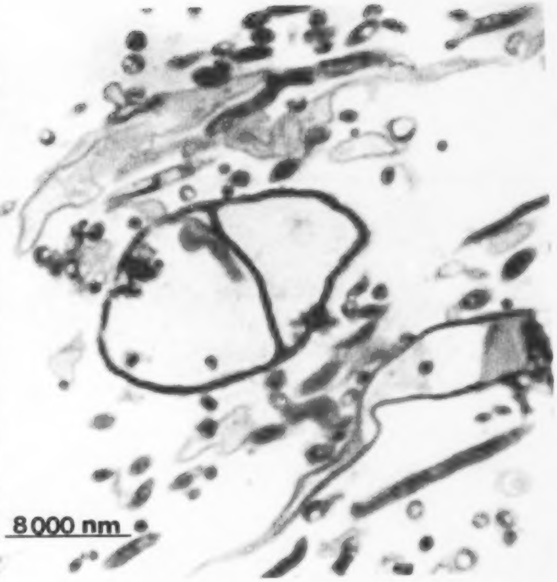
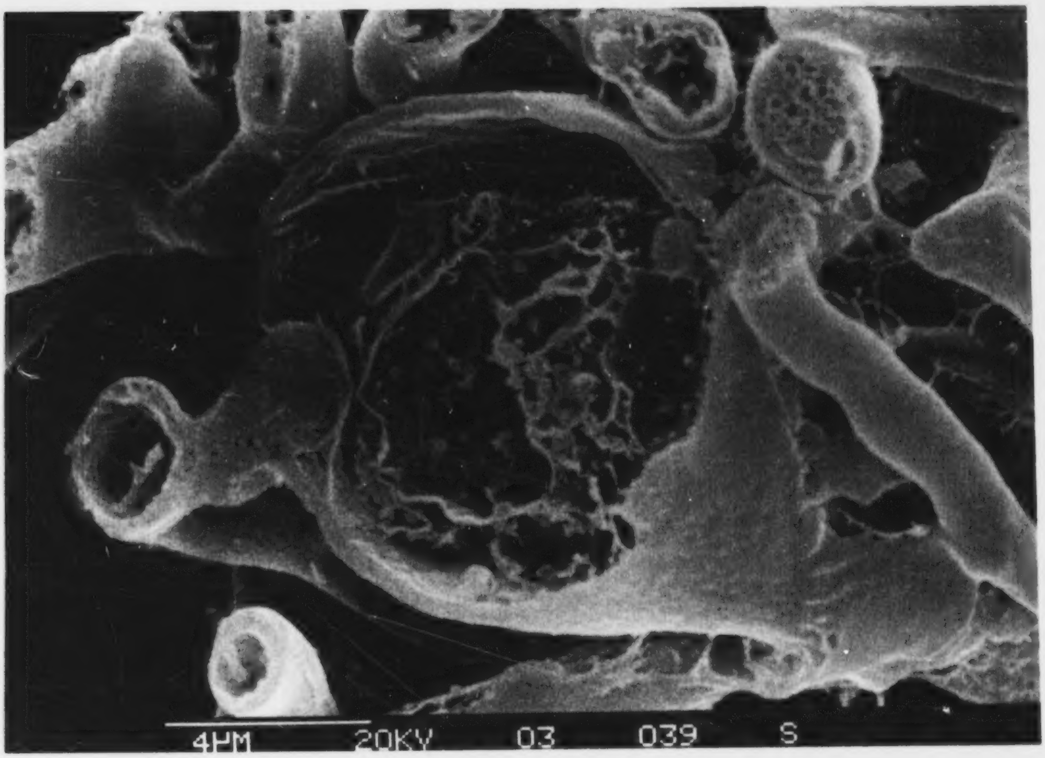

8000 nm

Figure 3.36. Scanning electron micrograph of *Gliocladium roseum* penetrating a conidium of *Alternaria brassicae* after 48 hours (top). Note that the cell wall of *A. brassicae* is not pushed inwards, indicating enzymatic penetration. *Alternaria brassicae* conidium showing a perforation where *G. roseum* penetrated the conidium after 48 hours (bottom). The *G. roseum* hypha was dislodged.


4µM
20KV
03
039
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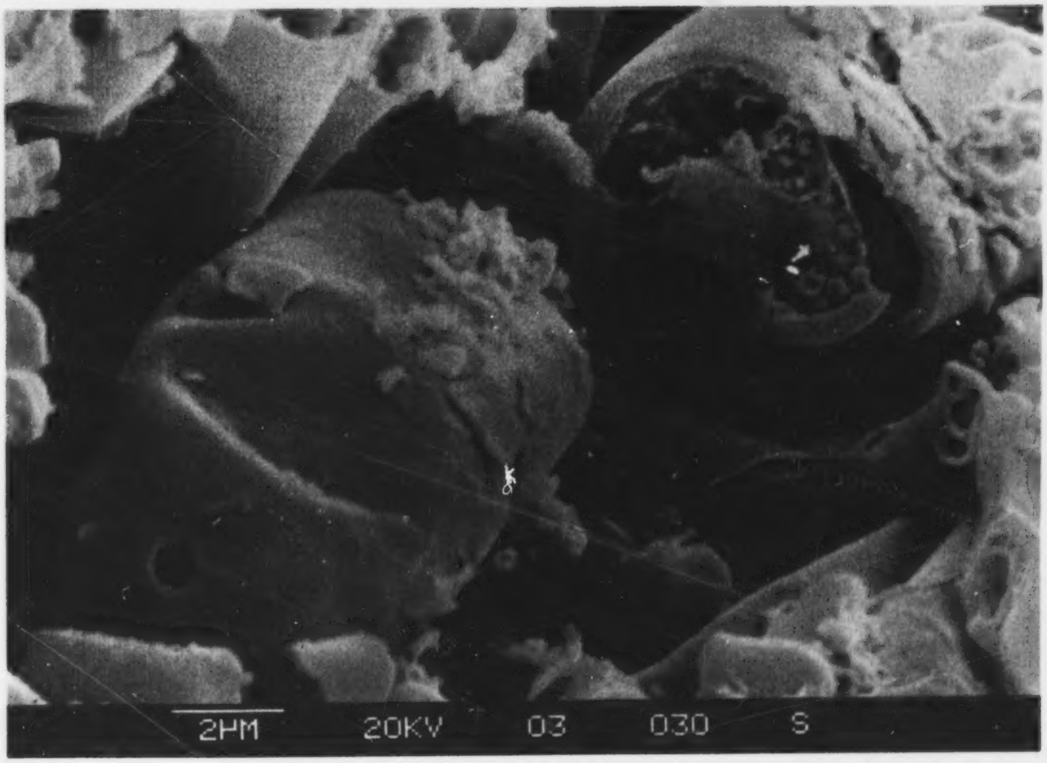
2µM
20KV
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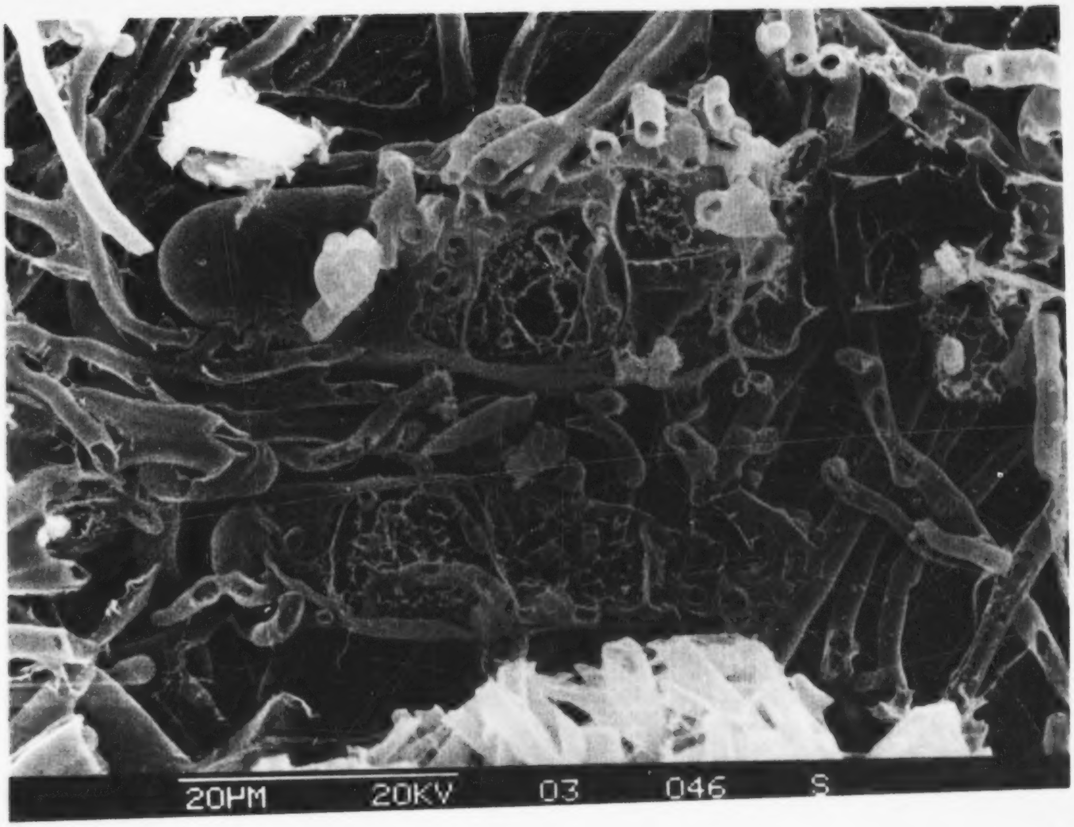
S

Figure 3.37. A scanning electron micrograph of an overview of *Alternaria brassicae* conidia parasitized by *Gliocladium roseum.* Note the hyphae of *G. roseum* within the *A. brassicae* conidium.


20µM
20KV
03
046
S

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CHAPTER FOUR: EFFECTS OF GLIOCLADIUM ROSEUM AND MYROTHECIUM VERRUCARIA TREATMENTS ON THE DEVELOPMENT AND SEVERITY OF ALTERNARIA BLACKSPOT

Introduction

Disease control in the field is usually obtained through a combination of chemical and cultural control practices. If practiced properly, these measures can effectively control disease. Due to increasing concerns about the effects of chemicals on non-target organisms and chemical residues in soil and ground water, biological control is an attractive option. Both phylloplane (Tsuneda, 1977) and soil organisms have been studied as potential biological control organisms for the phylloplane. A series of papers published in the era of 1920-1930 was the first report of the direct use of soil micro-organisms to inoculate plants and reduce disease (Mostafa, 1993; Campbell, 1990). Unfortunately, in most instances biological control on the phylloplane is plagued with problems, not the least important of which is its lack of economic viability and problems with application of biocontrol agents (Lewis et al., 1989; Baker, 1986). Under buffered conditions, such as those in greenhouses, biological control on the phylloplane can be successful. Bélanger et al. (1994) successfully used *Sporothrix flocculosa*, a yeastlike fungus, to control rose powdery mildew in the greenhouse. However, the phylloplane in the field is a very unstable environment, thus rendering biological control on the phylloplane under field conditions very difficult. There are numerous reports of successful biocontrol in controlled environments, but failure of control in the field was common. One such example is the reduction of foliar symptoms of *Helminthosporium maydis* (Nisikado & Miyake) on maize under controlled conditions, but not in the field (Slessman and Leben, 1976). Through integration of chemical, cultural and biological control a new era of disease management may emerge (Baker, 1986). This chapter deals with gaining an understanding of the basic relationships of *Gliocladium roseum* Bainier, *Myrothecium verrucaria* (Alb. & Schw.) Ditmar. and *Alternaria brassicae* (Berk.) Sacc. on leaves of two cultivars of canola. The knowledge obtained here may be of use in future studies of biological control of Alternaria Blackspot on the phylloplane.

Materials and Methods

Growth Conditions

Canola cultivars Legend (*Brassica napus* L.) and Reward (*Brassica rapa* L.) were grown in a growth chamber at 20°C daytime and 18°C nighttime with a 16 hour photoperiod provided by F96T12 cool white VHO fluorescent bulbs. A light intensity of approximately 300 µE/m^2/sec was maintained at plant level. Six plants per pot were grown in 25 cm plastic pots containing a soil mix of 2:1 sterilized loam:metro mix and were watered every 2 weeks with a 20:20:20 fertilizer.

General Materials and Methods for Excised Leaf Tests

For all experiments, leaves of Legend and Reward were used at growth stage 3.1 to 3.2 (Harper and Berkenkamp, 1975). For all excised leaf experiments, the third and/or fourth true leaves were placed in sterile moist chambers which consisted of 15 cm diameter glass petri plates containing moistened paper towel. Droplets of spore suspension were placed on each half of the leaf, with the midrib used as the dividing line. One droplet being a water control, one an *A. brassicae* control, one an antagonist control or a control for metabolites of *M. verrucaria* and one a combination of *A. brassicae* with antagonist or metabolites of *M. verrucaria*, respectively. Treatments consisted of *A. brassicae* alone and in combination with any of: 1) unwashed conidia of *M. verrucaria*; 2) washed conidia of *M. verrucaria,* 3) metabolites of *M verrucaria* ; 4) conidia of *G. roseum* isolate 98; or 5) conidia of *G. roseum* isolate 17. Treatment controls included washed and unwashed conidia of *M. verrucaria* and metabolites *of M. verrucaria* as well as conidia of *G. roseum* isolates 98 and 17 in isolation. Sterile distilled water was used as an overall control. Droplet positions were rotated on the leaf to avoid any bias. The final spore concentrations in each 40 µL droplet for both *G. roseum* isolates, washed and unwashed conidia of *M. verrucaria* and *A. brassicae* were 2.0×10^5, 6.5×10^6 and 2.5×10^5 spores/mL, respectively.

After inoculation, the leaves were observed for disease symptoms daily for six days. Disease severity was recorded on a scale of 0 to 5 with zero showing no symptoms and 5 indicating severe disease which appeared as bright yellow lesions. Each type of leaf test was conducted three times with the exception of the leaf germination test which was conducted twice. These experiments were conducted at room temperature with leaves being exposed to a light-dark cycle. Moist chambers containing leaves were arranged randomly.

For all lesion severity graphs, the lesion severities of 0 to 5 were converted to percentages and ranked within plant and within treatment.

Germination on Excised Leaves

Arrangement of droplets for this test was as previously described with each droplet being 40 µL. Droplets which contained combinations of fungi or *A. brassicae* plus metabolites of *M. verrucaria* were prepared in combination so that the fungi would be applied simultaneously. After 2, 4, 6 and 8 hours, 2 leaf pieces approximately 1 cm x 1 cm were cut (each from a different inoculated leaf at a different position) for each treatment and floated on 70% ethanol at room temperature until they cleared. Leaf pieces were mounted on glass slides, stained with lactophenol cotton blue and observed. A total of one hundred conidia of each fungus on each leaf piece for each treatment were observed for germination. In this case, time zero was considered to be half way through the time required to place all of the droplets on the leaves. This was usually when *A. brassicae* was placed on the leaves.

In order to determine if germination seen on the leaf pieces was affected by the leaves or not, germination of all fungi was also observed on glass slides. The same time intervals were used for sampling and time zero was as previously described. Treatments for the slide test were similar to those for the leaf test, however, only one 40 µL droplet of spore suspension was placed on each slide. Two slides were observed for each treatment at each time as previously described. This experiment was conducted once.

Hyphal Interaction on Leaves

Droplets of conidial suspension were applied to leaves of Legend and Reward as previously described. At 2 and 4 days post-inoculation, leaf pieces approximately 1 cm x 1 cm were cut from these leaves and treated as previously described. After the leaf pieces had cleared, they were observed for parasitism. Observations for parasitism were also made on leaf pieces from *in situ* leaves of Legend and Reward.

Alternaria brassicae and Antagonists Applied Simultaneously

Droplets of spore suspension were placed on leaves as previously described (see germination on leaves). Four leaves were used for each antagonist treatment (twenty leaves total) with 8 droplets per leaf.

Alternaria brassicae Applied Prior to Application of Antagonists or Metabolites of *M. verrucaria*

Treatments were as previously described. Twenty microlitre droplets of *A. brassicae* were applied in the morning and 10 hours later, 20 µL droplets of antagonists or metabolites of *M. verrucaria* were applied to *A. brassicae* droplets or water droplets in order to achieve the aforementioned concentrations.

Antagonists and Metabolites of *M. verrucaria* Applied Prior to Application of *Alternaria brassicae*

Twenty microlitre droplets of antagonists were applied to leaves as previously described. *Alternaria brassicae* was applied to antagonist droplets, droplets of metabolites of *M. verrucaria* or water droplets 10 hours after application of the antagonists and metabolite. Again, final concentrations were the same as previously described.

Wax Test

Leaves of Legend were used for this experiment because it is the waxier of the 2 cultivars used. A sterile cotton ball, moistened with sterile distilled water was used to disturb the wax on the left half of the leaf by gently rubbing the leaf from tip to base. Droplets of fungal suspension were applied as previously described.

In situ Plant Tests

Plants were thinned to 3 plants per pot with the third true leaf of each plant being used. The midrib was used as the dividing point with one half of each leaf being treated with *A. brassicae* only and the other half with *A. brassicae* in combination with the antagonist or metabolites of *M. verrucaria*. For plants used as control, one half of the leaf was treated with antagonist only or metabolites of *M. verrucaria* only and the other half with sterile distilled water. Ten 10 µL droplets were placed on the leaf, spread with the pipette tip, and 10 more 10 µL droplets were placed on the leaf (total of 200 µL per half leaf). Treated leaves were covered with polyethylene bags to prevent the droplets from drying quickly. Leaves were sprayed with sterile distilled water daily and were observed daily for disease symptoms for 4 days post-inoculation. Plants used in these tests were used at the same growth stage and were rated with the same disease scale as previously described. This experiment was conducted 3 times. The same spore concentrations were used for *in situ* plant tests as for excised leaf tests. The term *in situ* is used as opposed to intact because intact could refer to an excised whole leaf, whereas *in situ* refers to a whole leaf attached to the plant.

Statistical Analyses

Analysis of variance was used to determine differences of lesion severity and days to appearance of disease and were computed using GLM on SAS. A least significant difference procedure (t test) was used. Alpha equal to 0.05 was used to decide if differences were significant.

Results

Germination on Leaves

In all cases, *A. brassicae* germinated in advance of the conidia of both isolates of *G. roseum* and washed and unwashed conidia of *M. verrucaria* on the leaves of Legend and Reward and on glass slides (Figures 4.1 and 4.2). After 2 hours, *A. brassicae* combined with *G. roseum* isolate 17 had greater germination than the *A. brassicae* control on leaves of Legend and Reward and in the slide test. Percent germination of the controls was 33.75, 33.75 and 8.5, respectively, while that for *G. roseum* isolate 17-treated *A. brassicae* was 37.5, 51.25 and 14.5 percent, respectively. Germination of *A. brassicae* was reduced by the 3 *M. verrucaria* treatments. *Alternaria brassicae* control germination on the leaves of Legend and Reward and on glass slides, after 8 hours, was 73.5, 80.25 and 48.5, respectively. When *A. brassicae* conidia were combined with washed conidia of *M. verrucaria*, the percent germination of *A. brassicae* on leaves of Legend and Reward and on glass slides was 12.25, 23.5 and 8.5, respectively. Upon observation of *A. brassicae* conidia in combination with unwashed conidia of *M. verrucaria* on leaves of Legend, Reward and on glass slides, the percent germination of *A. brassicae* was 0.0. When *A. brassicae* conidia were combined with metabolites of *M. verrucaria* on leaves of Legend and Reward and on glass slides, the percent germination of *A. brassicae* was 57.5, 60.75 and 35.0, respectively. Germination of *A. brassicae* when combined with both isolates of *G. roseum* was nearly the same as that for the control. Percent germination of *A. brassicae* on leaves of Legend and Reward and on glass slides after 8 hours when combined with *G. roseum* isolates 98 and 17 was 73.25, 72.0 and 46.5 and 71.25, 76.0 and 48.0, respectively.

The percentage germination of both *M. verrucaria* and *G. roseum* were greater when they were in association with *A. brassicae* (Figures 4.1 and 4.2) After 8 hours the percent germination for controls of washed and unwashed conidia of *M. verrucaria* on leaves of Legend and Reward and on glass slides were 5.25, 7.0 and 10.5 and 25.5, 19.25 and 22.0, respectively. When combined with *A. brassicae* the percent germination for washed and unwashed conidia on Legend and Reward leaves and on glass slides was 48.00, 41.75 and 36.5 and 71.25, 70.25 and 48.0, respectively. The same trend was noted for *G. roseum* germination. On leaves of Legend and Reward and on glass slides the percent germination for controls for *G. roseum* isolates 98 and 17 were 76.5, 77.0 and 55.0 and 50.25, 53.25 and 62.0, respectively. When in combination with *A. brassicae*, the percent germination for *G. roseum* isolates 98 and 17 on leaves of Legend and Reward and on glass slides was 75.25, 86.5 and 73.0 and 78.25, 57.0 and 91.0, respectively.

The similarity of the results obtained from the leaves of Legend and Reward indicated that germination of the fungi was not affected by the leaves. This suggested that the observed germination was germination in the water droplet, an observation that was confirmed by the results of the slide germination test.

Hyphal Interaction on Leaves

Leaf pieces from both *in situ* and excised leaves of Legend and Reward were observed by light microscopy 2 and 4 days post-inoculation. It was noted that when the *A. brassicae* conidia were combined with the unwashed conidia of *M. verrucaria*, very little growth of *A. brassicae* occurred (Figure 4.3), while there was profuse growth of *M. verrucaria*. Unlike unwashed conidia of *M. verrucaria*, the washed conidia had no visible effect upon the growth of *A. brassicae* . Both *A. brassicae* and *M. verrucaria* grown from washed conidia grew abundantly on the leaf surface. There was no parasitism of *A. brassicae* by *M. verrucaria* hyphae grown from either washed or unwashed conidia

(Figure 4.3). Metabolites of *M. verrucaria* did not affect the growth of *A. brassicae* when compared to the control. Upon observation of *A. brassicae* combined with either isolate of *G. roseum*, slight to abundant growth of *A brassicae* and both *G. roseum* isolates was seen (Figure 4.4), however, there was no parasitism of *A. brassicae* by either isolate of *G. roseum* (Figure 4.4).

Alternaria brassicae and Antagonists Applied Simultaneously

Alternaria brassicae in combination with washed conidia of *M. verrucaria* resulted in disease symptoms occurring sooner (0.98 days) on leaves of Legend than disease symptoms caused by *A. brassicae* alone (Table 4.1). There was no significant difference in days to appearance of disease for leaves of Reward. For both Legend and Reward, appearance of disease symptoms was delayed by 2.40 days and 1.59 days, respectively. Appearance of disease symptoms was not significantly different on Legend and Reward when *A. brassicae* was combined with metabolites of *M. verrucaria* .

Neither isolate of *G. roseum* had any affect on the appearance of disease symptoms caused by *A. brassicae* on either of the canola cultivars (Table 4.1).

Lesions caused by *A. brassicae* on Reward leaves were more severe 4 days post-inoculation than were lesions formed with *A. brassicae* in combination with washed conidia of *M. verrucaria* (Figure 4.8). No significant difference in lesion formation was noted on any of the other days (Figure 4.9). On leaves of Legend, washed conidia of *M. verrucaria* in combination with *A. brassicae* resulted in formation of more severe lesions 3 and 4 days post-inoculation than did *A. brassicae* alone (Figure 4.5). There was not a significant difference in lesion severity on the other days (Figure 4.6). On leaves of Reward, lesions caused by *A. brassicae* were much more severe than lesions caused by *A. brassicae* in combination with unwashed conidia of *M. verrucaria* 3, 4, 5, and 6 days post-inoculation (Figure 4.9). For the same treatment on leaves of Legend, the same results were seen starting at 2 days post-inoculation (Figures 4.5, 4.8 and 4.6). Metabolites of *M. verrucaria* did not significantly decrease lesion severity on the leaves of Reward (Figure 4.10). In fact, 4 days post-inoculation, lesion severity of *A. brassicae* with metabolites of *M. verrucaria* was greater than lesion severity of *A. brassicae* alone. This may be due in part to the phytotoxic nature of some of the trichothecenes. Lack of reduction of severity of lesions was also seen on Legend leaves 4, 5, and 6 days post-inoculation (Figure 4.7), however, *A. brassicae* itself formed more severe lesions two and three days post-inoculation (Figures 4.5 and 4.8).

On the leaves of Reward, lesions caused by *A. brassicae* were more severe 4, 5 and 6 days post-inoculation than were lesions caused by *A. brassicae* in combination with the conidia of *G. roseum* isolate 98, while on leaves of Legend, lesions caused by *A. brassicae* were more severe 5 and 6 days post-inoculation (Figures 4.11-4.14). Two and 3 days post-inoculation, *A. brassicae* in combination with *G. roseum* isolate 17 had greater severity ratings than *A. brassicae* alone on the leaves of Reward. Four, 5 and 6 days post-inoculation, lesion severity was similar (Figure 4.14). Lesion severity of *A. brassicae* with *G. roseum* isolate 17 on Legend was greater 3 days post-inoculation than was severity of lesions caused by *A. brassicae* alone. Four, 5 and 6 days post-inoculation, lesion severity was similar (Figures 4.11-4.13).

No disease symptoms were caused by the distilled water droplets or any of the antagonist treatments or metabolites of *M. verrucaria*.

When dried, senesced leaves of Legend and Reward were observed, it was noted that *G. roseum* isolates 98 and 17 had overgrown *A. brassicae* where the fungi were applied in combination. As a result, both isolates of *G. roseum* prevented sporulation of *A. brassicae*.

Alternaria brassicae* Applied Prior to Application of Antagonists and Metabolites of *M. verrucaria

On excised leaves of Legend and Reward, metabolites of *M. verrucaria* and washed conidia of M. verrucaria did not significantly delay the days to appearance of disease symptoms caused by *A. brassicae* on either canola cultivar. Unwashed conidia of *M. verrucaria* significantly delayed the onset of disease symptoms on both cultivars (Table 4.2).

Neither isolate of *G. roseum* delayed the onset of disease symptoms. In fact, disease symptoms were observed earlier when *A. brassicae* was combined with *G. roseum* isolates, but the difference was not significant (Table 4.2).

Three, 4 and 5 days post-inoculation, lesion severity on the leaves of Legend was significantly reduced when washed conidia of *M. verrucaria* were applied to the conidia of *A. brassicae* (Figures 4.15 and 4.16). Two, 4, 5 and 6 days post-inoculation, washed conidia of *M. verrucaria* in combination with *A. brassicae* resulted in a reduction of lesion severity on Reward leaves (Figures 4.18 and 4.19). There is no clear explanation for the results obtained on the third day post-inoculation. Lesion severity on leaves of Legend and Reward was significantly reduced 2, 3, 4, 5, and 6 days post-inoculation by application of unwashed conidia of *M. verrucaria* (Figures 4.15, 4.18, 4.16 and 4.19). Metabolites of *M. verrucaria* had no significant affect on lesion severity on leaves of Legend or Reward (Figures 4.17 and 4.20), except on the second day post-inoculation when *A. brassicae* lesions were less severe than the lesions caused by A. *brassicae* in combination with metabolites of *M. verrucaria* on leaves of Legend (Figures 4.15 and 4.18). In some cases, lesion severity was slightly, but not significantly enhanced (Figure 4.18). This may be due in part to the phytotoxic nature of some trichothecenes.

Gliocladium roseum isolate 98 had no affect on lesion severity on the leaves of either cultivar (Figures 4.21-4.24). Lesion severity on leaves of Reward was not reduced by application of *G. roseum* isolate 17 (Figure 4.24), however, 5 days post-inoculation *A. brassicae* lesions were less severe on Legend leaves (Figures 4.21-4.23). There is no clear explanation for this. On all the other days, the severity of lesions of *A. brassicae* and *A. brassicae* in combination with *G. roseum* isolate 17 were not significantly different. It is not clearly understood why there was no difference of lesion severity on day 6.

No symptoms were caused by the distilled water droplets or any of the antagonist treatments or metabolites of *M. verrucaria*.

Antagonists and Metabolites of *M. verrucaria* Applied Prior to Application of *Alternaria brassicae*

In general, leaves of Reward senesced rapidly. The data presented for all treatments on the leaves of Reward are therefore not as accurate as the data presented for the other experiments. Once the leaves treated with the washed and unwashed conidia of *M. verrucaria* turned chlorotic, *M. verrucaria* grew and sporulated on the leaf surface. Due to the rapid senescence of *M. verrucaria* inoculated leaves, *M. verrucaria* damage on the leaves which interfered with appearance of lesions caused by *A. brassicae* and the subsequent lack of accuracy of the data, the data for days to appearance of disease when *A. brassicae* was combined with *M. verrucaria* has been removed from Table 4.3. Results of lesion severity are presented but are believed to be somewhat inaccurate. Rapid chlorosis of the leaves treated with metabolites of *M. verrucaria* and *G. roseum* isolates 98 and 17 may also have skewed that data somewhat, however there was no interference of *A. brassicae* disease symptoms by those treatments so the results for days to appearance of disease are presented. Lesion severity data (Figures 4.28 and 4.33) may also be somewhat skewed due to a drastically decreased number of observations for each replicate in the experiment.

On leaves of Legend, application of unwashed conidia of *M. verrucaria* prior to *A. brassicae* conidia significantly delayed (1.23 days) the onset of disease symptoms caused by *A. brassicae* (Table 4.3). Washed conidia of *M. verrucaria* and metabolites of *M. verrucaria* had no effect upon days to appearance of disease symptoms on the leaves of Legend (Table 4.3). On leaves of Reward, metabolites of *M. verrucaria* did not delay the days to appearance of disease symptoms caused by *A. brassicae* (Table 4.3).

Gliocladium roseum isolate 17 significantly delayed days to appearance of disease symptoms (0.91 days) on leaves of Legend, while isolate 98 did not (Table 2.3). Neither isolate of *G. roseum* delayed the appearance of Alternaria Blackspot symptoms on leaves of Reward (Table 4.3).

Application of washed conidia of *M. verrucaria* prior to application of *A. brassicae* did not affect lesion severity on leaves of Legend at any time (Figures 4.25 and 4.26). Lesion severity on leaves of Reward was not significantly different at any time (Figures 4.28 and 4.29). However, the lesion severity for *A. brassicae* combined with washed conidia of *M. verrucaria* was due mainly to disease symptoms caused by *M. verrucaria*. Very little if any of the symptoms caused by infection by *A. brassicae* were noted on Reward for this combination in this experiment. No lesion severity rating is presented for the sixth day post-inoculation because all leaves senesced and lesions were unratable by that time (Figure 4.28). Three, 4, 5 and 6 days post-inoculation, lesions caused by *A. brassicae* in combination with the unwashed conidia of *M. verrucaria* were significantly less severe than the lesions caused by the *A. brassicae* control on leaves of Legend (Figures 4.25 and 4.26). The combination of *M. verrucaria* unwashed conidia with *A. brassicae* on leaves of Reward behaved the same as the washed conidia of *M. verrucaria* (Figure 4.29). No difference in lesion severity was noted on leaves of Legend and Reward when metabolites of *M. verrucaria* were applied prior to *A. brassicae* (Figures 4.25, 4.28, 4.27 and 4.30).

On leaves of Legend, 2 days post-inoculation, *A. brassicae* in combination with *G. roseum* 98 had more severe lesions than *A. brassicae* alone (Figure 4.31). Lesion severity was not significantly different 3, 4, 5, and 6 days post-inoculation (Figure 4.32). Application of *G. roseum* isolate 98 to *A. brassicae* on leaves of Reward did not result in any difference in lesion severity between this combination and the *A. brassicae* control (Figures 4.33 and 4.34). Day 6 lesion severity results for Reward are not presented on the graph because all leaves senesced prior to day 6. On leaves of Reward, application of *G. roseum* isolate 17 prior to *A. brassicae,* did not significantly affect lesion severity at any time (Figures 4.33 and 4.34). This was not the case for the leaves of Legend on which lesion severity was greatly reduced 4, 5 and 6 days post-inoculation (Figure 4.31 and 4.32).

Neither *G. roseum* isolate, metabolites of *M. verrucaria* or the distilled water droplets caused any symptoms.

Wax Test

Rubbing the leaf surface with a cotton ball effectively damaged the wax on the leaf surface. This was evidenced by the improved retention of the droplets placed on the leaf.

Treatment of *A. brassicae* with washed conidia of *M. verrucaria* did not delay the days to appearance of disease for either wax treatment (Table 4.4). For both wax treatments, the combinations of *A. brassicae* and unwashed *M. verrucaria* significantly delayed the days to appearance of disease. Symptoms of *A. brassicae* occurred 2.22 and 2.65 days later for the undisturbed wax and disturbed wax treatments, respectively. No delay was seen when *A. brassicae* was combined with the metabolites of *M. verrucaria* (Table 4.4).

Gliocladium roseum isolate 98 delayed the days to appearance of disease caused by *A. brassicae* on the half of the leaf with undisturbed wax, but not on the half of the leaf where wax was disturbed (Table 4.4). There was no significant difference in the days to appearance of disease for either wax treatment when *A. brassicae* was combined with *G. roseum* isolate 17 (Table 4.4).

Lesion severity was the same for both wax treatments 5 and 6 days post-inoculation (Figure 4.37), when *A. brassicae* was combined with unwashed conidia of *M. verrucaria* (Figure 4.35). Severity of lesions was greater 2, 3, and 4 days post-inoculation on the half of the leaf where wax was disturbed. This increase in severity was not always significant. Three, 4, 5 and 6 days post-inoculation, lesion severity of *A. brassicae* combined with unwashed conidia of *M. verrucaria* (Figure 4.36) was significantly reduced (Figure 4.37) for both wax treatments and the severity of lesions was similar for both wax treatments. Lesion severity of *A. brassicae* combined with metabolites of *M. verrucaria* (Figure 4.38) and *A. brassicae* alone were significantly greater 3, 4, and 5 days post-inoculation for the disturbed wax treatment. Lesion severities became more even at 6 days post-inoculation (Figure 4.39).

Two, 3 and 4 days post-inoculation, lesion severity of the disturbed wax treatment was greater than for the undisturbed wax treatment (Figure 4.40). The disturbed wax and undisturbed wax treatments produced lesions of the same severity for *G. roseum* treated and untreated *A. brassicae* 2, 3 and 4 days post-inoculation. Five and 6 days post-inoculation, *G. roseum* treated *A. brassicae* lesions were significantly less severe than all other lesions in the two wax treatments (Figure 4.42). Two days post-inoculation, lesions were more severe for *A. brassicae* combined with *G. roseum* isolate 17 (Figure 4.41) for both wax treatments. Three, 4, 5 and 6 days post-inoculation, the disturbed wax treatment showed more severe lesions for both *A. brassicae* and *A. brassicae* with *G. roseum* isolate 17 (Figure 4.42). Lesion severity on these days was similar within wax treatment.

No symptoms were caused by the distilled water droplets or any of the antagonist treatments or metabolites of M. verrucaria.

In situ Plant Test

Unwashed conidia of *M. verrucaria* when combined with *A. brassicae* caused severe leaf damage on both Legend and Reward in the two replications of this experiment. *Myrothecium verrucaria* did not cause any disease symptoms in this combination the first time this experiment was carried out. Possible explanations for this are the leaves may have been damaged more when the conidia were applied in the replicates. Also, the plants may not have been as healthy or the spore concentration could have been higher than on the initial run. As this experiment was not conducted in a growth chamber, changes in the room conditions from the first time the experiment was carried out to the time that the replicates were run could have had an affect on the outcome of the experiment. When the overall control for this experiment was conducted, leaves combined with unwashed conidia of *M. verrucaria* showed disease symptoms. These symptoms occurred on both Legend and Reward 2 days after inoculation and became severe by 4 days post-inoculation. Due to the interference of unwashed *M. verrucaria* with the appearance of *A. brassicae* symptoms, results for this combination have been removed from Table 4.5

When *A. brassicae* was combined with the washed conidia of *M. verrucaria* and the metabolites of *M. verrucaria* there was no significant difference in the number of days to appearance of disease on either canola cultivar (Table 4.5). Neither isolate of *G. roseum* (Table 4.5) had an affect on the days to appearance of disease on either cultivar. The number of days to appearance for *A. brassicae/M. verrucaria* disease symptoms were 2.11 and 2.22 for Legend and Reward, respectively.

There was no difference in the severity of lesions on the leaves of Legend when *A. brassicae* was combined with either the washed conidia of *M. verrucaria* or the metabolites of *M. verrucaria* (Figures 4.43 and 4.44). Lesion severity was reduced when *A. brassicae* was combined with the unwashed conidia of *M. verrucaria* (Figures 4.43 and 4.44). Three days post-inoculation, *A. brassicae* lesion severity was significantly greater than that of *A. brassicae* in combination with washed conidia of *M. verrucaria* on leaves of Reward (Figure 4.45 and 4.46). Unwashed conidia of *M. verrucaria* and the metabolites of *M. verrucaria* did not reduce severity of lesions on leaves of Reward (Figure 4.45 and 4.46). Lesion severities presented for the *A. brassicae*-unwashed *M. verrucaria* treatments are skewed as the severities include the data for *M. verrucaria* severity.

Gliocladium roseum isolate 98 did not affect lesion severity on leaves of Legend or Reward (Figures 4.47, 4.49, 4.48 and 4.50). On leaves of Legend, *G. roseum* isolate 17 behaved identically to isolate 98 (Figures 4.47 and 4.48). Three days post-inoculation, lesion severity was reduced only slightly, but significantly, by *G. roseum* isolate 17 on leaves of Reward (Figure 4.49 and 4.50).

Alternaria brassicae combined with unwashed conidia of *M. verrucaria* was the only treatment in which the appearance of disease symptoms caused by *A. brassicae* were delayed. However *M. verrucaria* caused disease on Legend and Reward plants in two of three replications.

No symptoms were caused by washed conidia of *M. verrucaria* or metabolites of *M. verrucaria*, either isolate of *G. roseum* or distilled water.

Discussion

Germination of the conidia of *A. brassicae* occurred in advance of that of either isolate of *G. roseum* and washed and unwashed conidia of *M. verrucaria*. A likely explanation for this is that due to the size of *A. brassicae* conidia in comparison to that of *G. roseum* and *M. verrucaria*, many more endogenous nutrients would be present in the *A. brassicae* conidia. It is probably through the use of these endogenous nutrients that *A. brassicae* is able to germinate in advance of the other fungi. It is proven that endogenous nutrients can support the initial stages of germination of fungi (Blakeman, 1985; Brodie and Blakeman, 1975). It may also be that the conidia of the antagonists undergo a short period of dormancy before they begin to germinate.

After 2 hours, germination of the conidia of *A. brassicae* was enhanced when *A. brassicae* was in combination with *G. roseum* isolate 17. This suggests that perhaps *G. roseum* isolate 17 produces a metabolite or leaks nutrients which *A. brassicae* can use in addition to its own nutrient sources. However, *A. brassicae* germination was not enhanced at all observation times, indicating that enhanced germination is a short lived phenomenon. This may also have been a result of the time required to inoculate the leaves. This combination could have been on the leaf surface for more than 2 hours before the sample was taken.

Germination of *A. brassicae* was inhibited by all *M. verrucaria* treatments. This was obviously due to the presence of metabolites of *M. verrucaria*. A complete discussion of this phenomenon is given in the discussion in Chapter 3.

After 8 hours, both *G. roseum* isolates had no significant affect on the germination of *A. brassicae* conidia. This suggests that no inhibitory compounds are produced by either isolate of *G. roseum* and that by 8 hours, the enhancement effect of *G. roseum* isolate 17 had diminished.

The presence of *A. brassicae* conidia had a marked affect on the germination of the conidia of both isolates of *G. roseum* and on washed and unwashed conidia of *M. verrucaria*. Enhancement of germination of antagonists is common, especially when the host is a fungus such as *A. brassicae*, whose conidia are known to leak nutrients (Tsuneda and Skoropad, 1978). These nutrients may be used by the antagonist and aid in its germination. Pachenari and Dix (1980) reported that when *G. roseum* was grown on *Botrytis allii* mycelium, the spores of *G. roseum* were stimulated.

Germination patterns of all fungi were similar on the leaves of both cultivars. This indicated that the leaf was having no affect on germination of the fungi. As the germination trends were similar for the experiment conducted on glass slides it appeared that the germination seen was germination in the water droplet.

When hyphal interactions on leaves were observed, no parasitism of *A. brassicae* was noted. This is most likely due to the low nutrient status of the water droplet on the leaf surface. In Chapter 3, it was clearly demonstrated that a high nutrient food source resulted in greater parasitism of *A. brassicae*. The only nutrients available to the fungi in the water droplet are those which leak from the leaf and from the fungi themselves. This evidently does not result in a high enough nutrient level to induce parasitism.

The overall trend exhibited by washed conidia of *M. verrucaria* was that this treatment did not reduce the days to appearance or severity of lesions of *A. brassicae*. In those instances where disease severity was reduced the most likely explanation is that the small amount of inhibition of *A. brassicae* that did occur during germination was enough to delay the onset of infection and disease for a short period. In some instances, disease occurred earlier or was more severe. One possible explanation for this is that if the leaf was weakened or damaged prior to application of the fungi, *A. brassicae* could have invaded and established infection earlier. Another possibility is that the metabolites produced by *M. verrucaria* or *M. verrucaria* itself could have begun to damage the leaf thereby making penetration and infection easier for *A. brassicae*.

Overall, the only treatment that consistently reduced the days to appearance of disease symptoms and the severity of lesions caused by *A. brassicae* was the unwashed conidia of *M. verrucaria*. Once again, the effects seen can be attributed to the metabolites carried with these unwashed conidia. One exception is when antagonists were applied to leaves of Reward prior to application of *A. brassicae*. The days to appearance of disease and lesion severity observed for combinations of *A. brassicae* with washed and unwashed conidia of *M. verrucaria* was mainly due to *M. verrucaria* symptoms. Another species of *Myrothecium* (*M. roridum*) is known to be pathogenic on canola and other crucifers (Rai et al., 1974) as well as on other plants (Ali et al., 1988) such as red clover and alfalfa (Leath and Kendall, 1983) and muskmelon (Kuti et al., 1989; Bruton, 1982; McLean and Sleeth, 1961) and has the potential to be used as a mycoherbicide in Malaysia (Ismail, 1990). Tewari and Skoropad (1977) reported *M. roridum* as a potential pathogen of rapeseed and mustard in Alberta, while *M. verrucaria* has been reported to be parasitic on rapeseed in

Australia (Barbetti and Sivasithamparam, 1981). It may be that the combination of environmental conditions in the moist chamber and leaf age were conducive to *M. verrucaria* causing disease.

Usually when the antagonist is applied prior to its host (the plant pathogen), the plant is not damaged by the antagonist and disease incidence is reduced. Work with *E. purpurascens, Aureobasidium pullulans* (de Bary) G. Arnaud, and *Cladosporium cladosporioides* (Fresen.) G. A. De Vries in India has demonstrated that these organisms, which were isolated from brassicas, inhibit the disease caused by *A. brassicae.* The results of this work showed that when the spores of these fungi or their metabolites were placed individually or collectively on the leaves, there was inhibition of disease caused by *A. brassicae.* The greatest amount of inhibition occurred when these organisms or their metabolites were placed on the leaf surface before the pathogen (Rotem, 1994; Rai and Singh, 1980). It has also been found that pretreating *Brassica* spp. with a spore suspension of *Streptomyces rochei* Berger, Jampolsky and Goldberg or its diffusate successfully reduced infection by *A. brassicicola* (Wu and Lu, 1984; Sharma and Gupta, 1978). Mostafa (1993) found that application of *M. verrucaria* one day in advance of *Drechslera teres* (Sacc.) Shoemaker resulted insignificant reduction of leaf symptoms on barley plants.

As previously mentioned, problems with *M. verrucaria* causing disease were encountered on both cultivars of canola in the *in situ* leaf test. However, unlike in the excised leaf test, only unwashed conidia of *M. verrucaria* caused disease in the *in situ* plant test. It is again possible that the combination of environmental conditions and leaf age may have been conducive to disease formation. Also implicated in the appearance of disease symptoms caused by *M. verrucaria* and the rapid chlorosis of the leaves are the metabolites themselves which are produced by *M. verrucaria.* The metabolites produced by *M. verrucaria* belong to a class of mycotoxins called the trichothecenes. Based on circumstantial evidence it is possible that the trichothecenes play a role in plant pathogenesis. Evidence includes a correlation between virulence and the ability to produce toxins *in vitro* as well as isolation of toxins from diseased plant tissues. Even when treated with very low concentrations of trichothecenes (10^{-5} to 10^{-6} M), a wide variety of plants show chlorosis, necrosis and other symptoms (Desjardins, 1992).

Metabolites of *M. verrucaria*, at the concentrations used in these studies, did not significantly reduce days to appearance of disease or lesion formation by *A. brassicae*. When *A. brassicae* was combined with the metabolites of *M. verrucaria* there was usually similarity of lesions between the combination and the *A. brassicae* control. In some instances lesion severity was reduced while in others it was enhanced. Reduction in lesion severity is most likely due to the the effects of the metabolites of *M. verrucaria* on *A. brassicae*. However the effectiveness of the metabolites of *M. verrucaria* as used was not great. A more reasonable explanation for the reduction in severities is that different leaves varied in the amount of wax present on the surface. These differences in wax characteristics can greatly affect disease development and severity. The reasons for the similarity of lesion severity are three-fold. Firstly, the concentration of metabolites was dilute in comparison to that carried on unwashed conidia of *M. verrucaria*. Secondly, unlike the situations where the conidia of *M. verrucaria* are present and producing metabolites, no additional metabolites were produced when only the metabolites of *M. verrucaria* were applied to the leaf. Thirdly, some of the trichothecene metabolites may have been converted into less toxic compounds by the leaves of plants.

The dilute nature of the metabolites in this situation is the main reason that lesion severity was not reduced. Even though germination of *A. brassicae* conidia was reduced, it was still great enough to cause disease. The continued production of metabolites as *M. verrucaria* grows is a possibility that should be considered, but it is not likely to have a great impact upon germination over a short time period. Even though the differences were not significantly different when washed conidia of *M. verrucaria* were combined with *A. brassicae*, there was a slight reduction in severity. Even this slight reduction was not possible when metabolites of *M. verrucaria* were combined with *A. brassicae* because the conidia of *M. verrucaria* were not present and producing metabolites. The idea of metabolites of *M. verrucaria* being transformed to other compounds is unlikely but is a possibility. If they were transformed to less toxic or non-toxic compounds, there would be very little or no effect on *A. brassicae*, resulting in a lack of difference between the lesion severities.

Enhancement of lesions may have been the result of cellular leakage from the canola leaf. Kuti et al. (1989) reported that with increasing concentration of trichothecenes produced by *M. roridum*, there was increased nutrient leakage from muskmelon leaves. If this were the case it would result in more nutrients being available to *A. brassicae* which would result in an increase in the severity of lesions caused by *A. brassicae*.

Gliocladium roseum isolate 98 did not significantly delay the days to appearance of symptoms caused by *A. brassicae*. In the one instance that there was a significant difference it was with the wax test. This particular instance demonstrated that development of disease was delayed when *A. brassicae* was combined with *G. roseum*. The most likely explanation for this is that the wax was undisturbed on that part of the leaf, demonstrating that wax had an affect on days to appearance of disease. Lack of reduction in severity of lesions is probably due to the fact that *G. roseum* isolate 98 does not inhibit *A. brassicae* germination. Therefore, disease severity would not be reduced. However, lesion severity was reduced at the later observation time when the combination of *A. brassicae* and *G. roseum* isolate 98 was applied simultaneously to excised leaves. A possible explanation for this is that *G. roseum* overgrew *A. brassicae* in the water droplet and the competition for nutrients was so great that the more effective saprophyte would have grown better. This means that *A. brassicae* would not have had an adequate supply of nutrients and as a consequence the lesion severity was reduced. *Gliocladium roseum* is commonly found in the soil existing as a saprophyte.

Generally, *G. roseum* isolate 17 had no affect upon the days to appearance of disease symptoms caused by *A. brassicae*. The one exception to this was when the antagonist was applied to leaves of Legend prior to the application of *A. brassicae*. The delay in days to appearance may be due to *G. roseum* 17 establishing itself on the leaf surface prior to application of *A. brassicae* thus reducing the ability of *A. brassicae* to infect the leaves. As well, Legend is a waxy cultivar of canola and the wax layer would also have presented a barrier to *A. brassicae*. The trend of lesion severity with respect to *G. roseum* isolate 17 was similarity in the severity of lesions. In some instances, lesion severity was more intense with the combination of *G. roseum* isolate 17 and *A. brassicae*. This is likely due to the enhanced germination of *A. brassicae* seen in the leaf germination tests. *Alternaria brassicae* can infect leaves within 6 hours when it is growing normally by itself. With the enhanced germination, infection of leaves may have occurred more quickly and this would result in the formation of more severe lesions at the onset of disease development when compared with the *A. brassicae* control. *Gliocladium roseum* isolate 17 did appear to reduce lesion severity in some instances. This was perhaps due to a thicker wax layer on the leaves used.

Overall, *G. roseum* isolates 98 and 17 did not delay disease appearance or reduce disease severity caused by *A. brassicae*. This may be due to the absence of a food base which may be required by *G. roseum* (Keinath et al, 1991), or the strains of *G. roseum* used may not have been effective in this situation. Of five strains of *G. roseum* tested by Keinath et al. (1991) in studies with *Verticillium dahliae*, two were found to be ineffective, demonstrating that there is variability between strains in their ability to control pathogens. It may also stand to reason that the spore concentration used was too low to result in control of *A. brassicae*. Keinath et al. (1991) found that when higher rates of *G. roseum* were added to the soil more effective control of *V. dahliae* was achieved.

The results of the wax test indicated that severity of lesions and days to appearance of disease were more severe and occurred earlier on the portion of the leaf where wax had been disturbed. This suggests that disturbing the wax layer results in the leaves being more susceptible to disease. A waxy layer on the leaf helps to reduce the susceptibility of plants to *A. brassicae*. The wax results in an hydrophobic surface which has low water retention of water-borne inoculum. It results in a decrease in germination of conidia and fewer germ tubes are produced (Conn and Tewari, 1989). This in turn may result in less severe disease as was seen on the undisturbed half of the leaf for at least the first few days post-inoculation. Disease severity tended to become similar near the end of the observation time. This may be due to the fact that disease was established in both wax treatments and once established on the undisturbed half of the leaf symptom development and severity occurred quickly enough to match that of the already more severe symptoms seen on the half of the leaf on which the wax was disturbed. One reason that disease development occurred more quickly and therefore was more severe at the early observation times on the leaf half with wax disturbed is because disturbing the wax results in increased germination of *A. brassicae* which may be due to an increase in leaf exudates (Conn and Tewari, 1989) caused by leaf damage when the wax was disturbed. Leaf damage resulting from disturbing the wax layer would result in the leaf being more susceptible to infection by *A. brassicae*. This is another possible explanation for the increase in disease severity and the earlier appearance of disease symptoms on the leaf where wax was removed. However, Conn (1986) reported that when wax is wiped from leaves, there appeared to be no mechanical damage to the epidermal cells of the leaf.

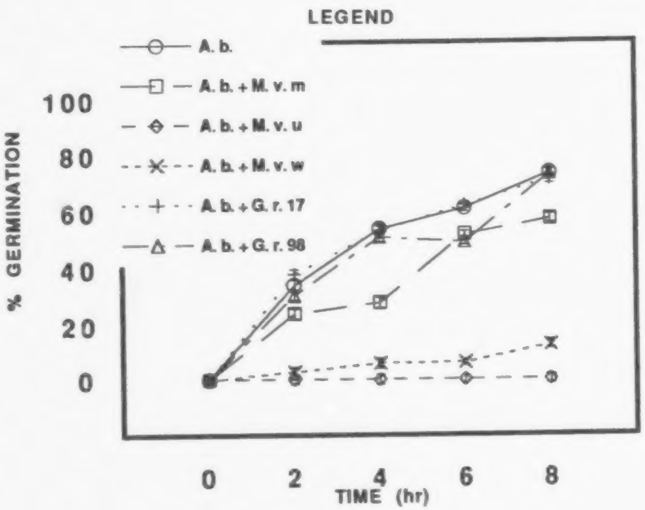
One interesting observation which was made in these studies is that disease symptoms appeared faster and were more severe on the *in situ* leaves. Generally, the opposite is true because it is believed that *in situ* leaves have better defence systems than excised leaves. One major difference between the two types of tests is that the droplet on the excised leaves rarely dried up because the leaves were placed in moist chambers. In those instances where droplets did dry quickly, disease appeared quickly and was more severe. With the *in situ* plant tests, even though the leaves were covered with plastic bags in an attempt to keep conditions humid, most of the droplets did dry overnight. Every day the *in situ* leaves were moistened, therefore they were subjected to a wetting and drying cycle which may be a cycle that is more conducive to disease development than is a constantly humid environment. Another possible explanation is the difference in inoculation techniques for the leaves in the two types of tests. It is possible that the wax layer on the *in situ* leaves was slightly damaged by the inoculation technique used. This would result in one of the defences of the leaves being reduced and therefore the leaves being made more susceptible to disease.

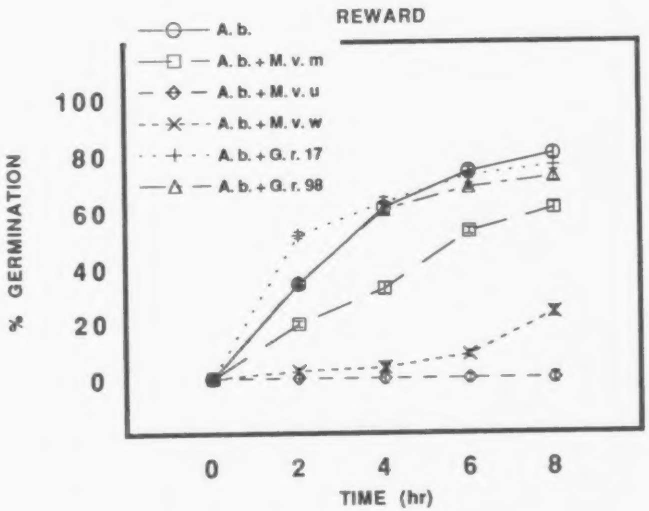
There is great potential for the use of *G. roseum* as a fall or early spring application to prevent the sporulation of *A. brassicae* on canola crop debris. It may even be that *G. roseum* will parasitize any existing conidia of *A. brassicae*, thereby reducing inoculum levels by killing conidia of *A. brassicae* which are already present and by inhibiting the formation of more conidia of *A. brassicae*.

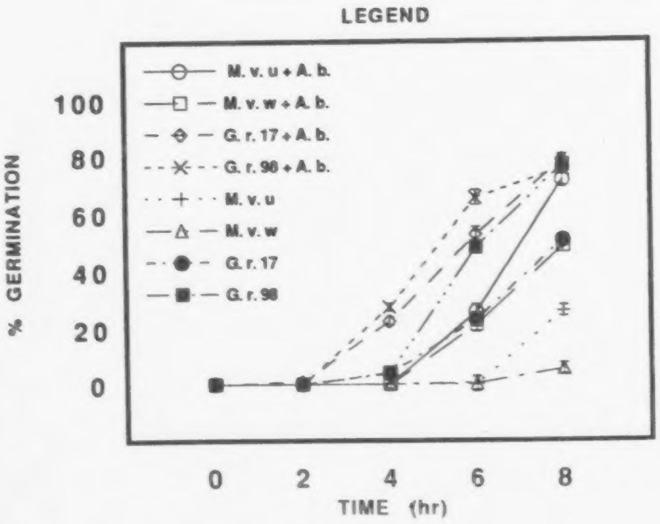
Conclusions

Myrothecium verrucaria conidia when unwashed effectively inhibit disease caused by *A. brassicae*. Washed conidia of *M. verrucaria* and metabolites of *M. verrucaria* were not as effective as the unwashed conidia. Only unwashed conidia of *M. verrucaria* showed any potential of being economically viable for use as biological control agents. The drawback being that *M. verrucaria* sometimes caused disease on leaves of Legend and Reward. *Gliocladium roseum* isolates 98 and 17 as used in these studies showed no potential for economical biological control of *A. brassicae* in the field.

Figure 4.1. Percent germination of the conidia of *Alternaria brassicae* alone and in combination with antagonists or metabolites of *Myrothecium verrucaria* on leaves of Legend (upper left) and Reward (upper right). Percent germination of the antagonists by themselves and when in combination with *A. brassicae* on leaves of Legend (lower left) and Reward (lower right). Error bars are present but the error was small enough that the error bars are difficult to see. (A. b. = *Alternaria brassicae*, M. v. = *Myrothecium verrucaria*, m = metabolites of *M. verrucaria*, u = unwashed conidia, w = washed conidia, G. r. = *Gliocladium roseum*, 98 and 17 refer to the isolates of *G. roseum*)


LEGEND
A. b.
A. b. + M. v. m
A. b. + M. v. u
A. b. + M. v. w
A. b. + G. r. 17
A. b. + G. r. 98
% GERMINATION
100
80
60
40
20
0
0
2
4
6
8
TIME (hr)


REWARD
A. b.
A. b. + M. v. m
A. b. + M. v. u
A. b. + M. v. w
A. b. + G. r. 17
A. b. + G. r. 98
% GERMINATION
100
80
60
40
20
0
0
2
4
6
8
TIME (hr)


LEGEND
M. v. u + A. b.
M. v. w + A. b.
G. r. 17 + A. b.
G. r. 98 + A. b.
M. v. u
M. v. w
G. r. 17
G. r. 98
% GERMINATION
100
80
60
40
20
0
0
2
4
6
8
TIME (hr)

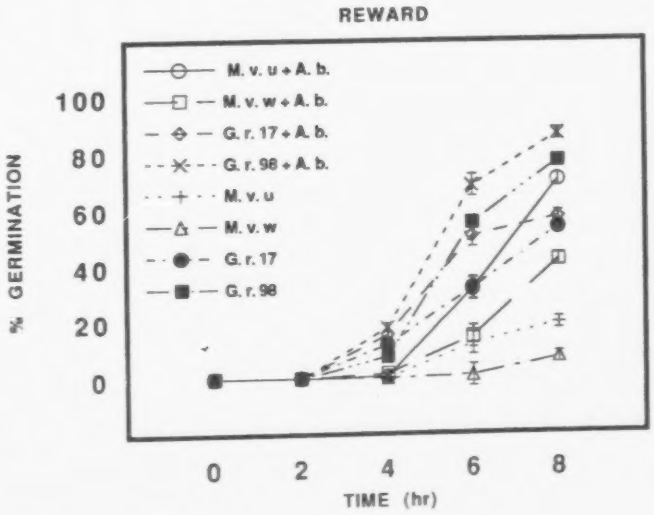
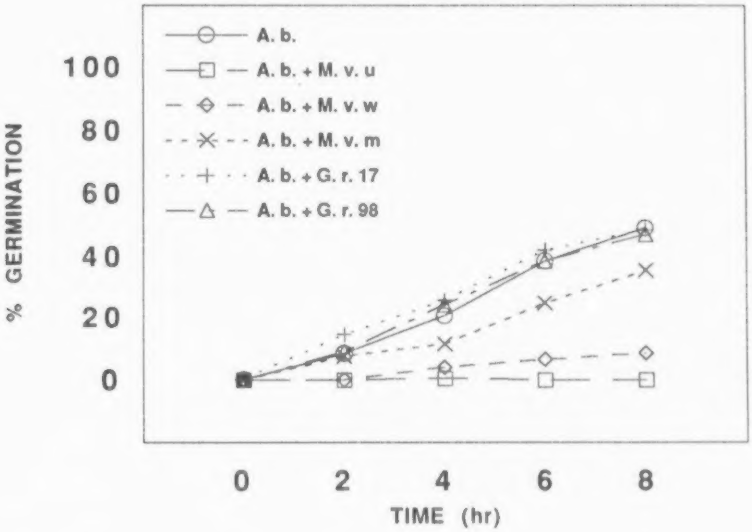

REWARD
M. v. u + A. b.
M. v. w + A. b.
G. r. 17 + A. b.
G. r. 98 + A. b.
M. v. u
M. v. w
G. r. 17
G. r. 98
% GERMINATION
100
80
60
40
20
0
0
2
4
6
8
TIME (hr)

Figure 4.2. Percent germination of the conidia of *Alternaria brassicae* alone and in combination with antagonists or metabolites of *Myrothecium verrucaria* on glass slides (top). Percent germination of antagonists by themselves and when in combination with *A. brassicae* on glass slides (bottom). (A. b. = *Alternaria brassicae*, M. v. = *Myrothecium verrucaria*, m = metabolites of *M. verrucaria*, u = unwashed conidia, w = washed conidia, G. r. = *Gliocladium roseum*, 98 and 17 refer to the isolates of *G. roseum*). As the experiment was carried out only once, no error bars are presented.


A. b.
A. b. + M. v. u
A. b. + M. v. w
A. b. + M. v. m
A. b. + G. r. 17
A. b. + G. r. 98
100
80
60
40
20
0
% GERMINATION
0
2
4
6
8
TIME (hr)

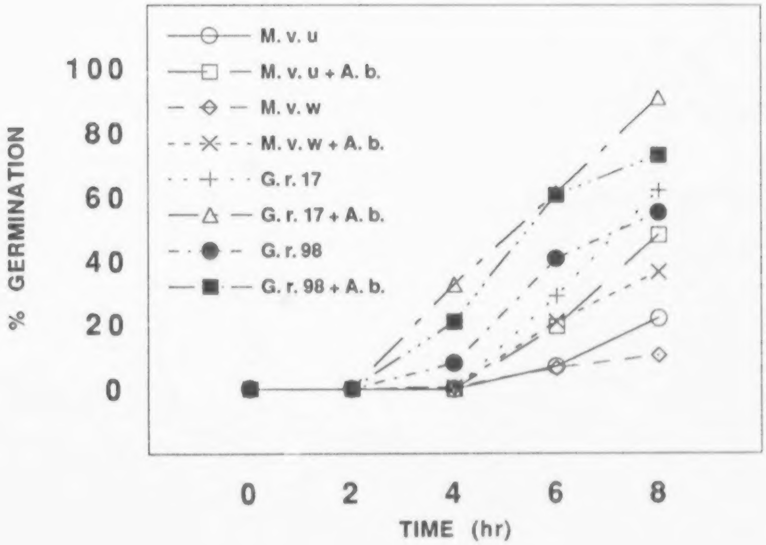

M. v. u
M. v. u + A. b.
M. v. w
M. v. w + A. b.
G. r. 17
G. r. 17 + A. b.
G. r. 98
G. r. 98 + A. b.
100
80
60
40
20
0
% GERMINATION
0
2
4
6
8
TIME (hr)

Figure 4.3. Hyphal interactions on leaves of Reward showing a lack of parasitism of *Alternaria brassicae* by *Myrothecium verrucaria* grown from washed conidia (top) and unwashed conidia (bottom) after 48 hours. (X 600)

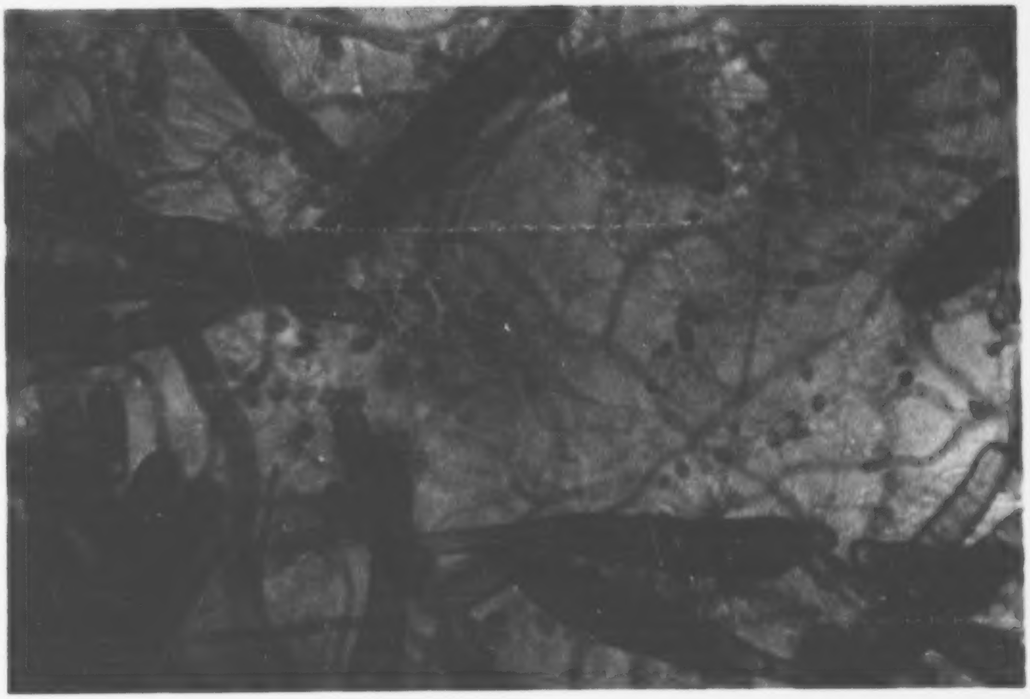
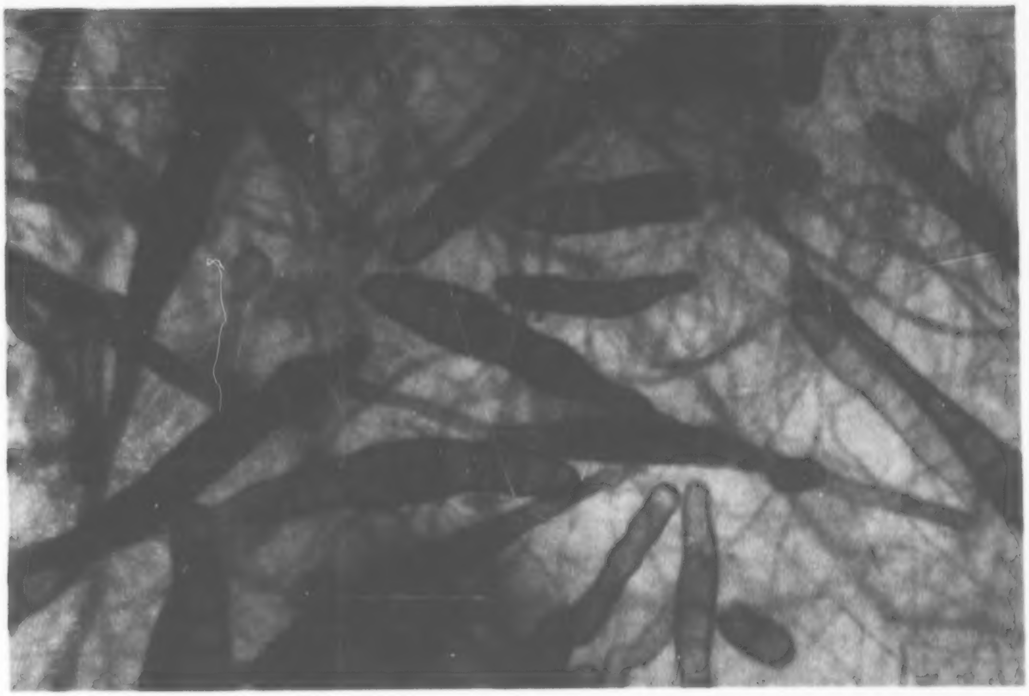


Figure 4.4. Hyphal interactions on leaves of Reward showing a lack of parasitism of *Alternaria brassicae* by *Gliocladium roseum* isolate 98 (top) and *G. roseum* isolate 17 (bottom) after 48 hours. (X 600)



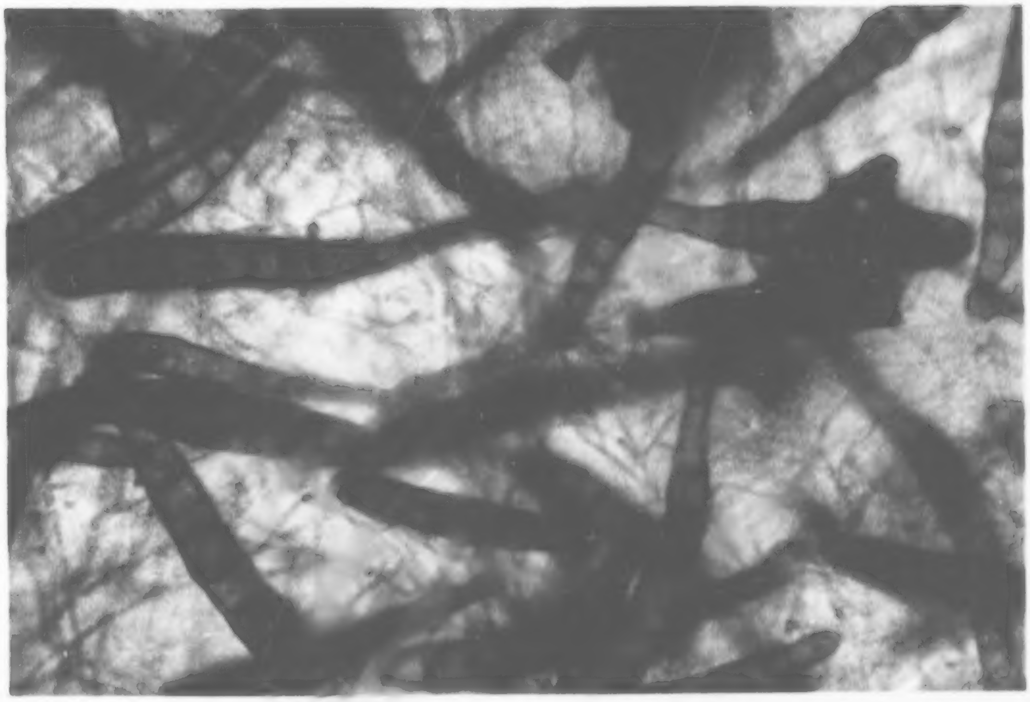
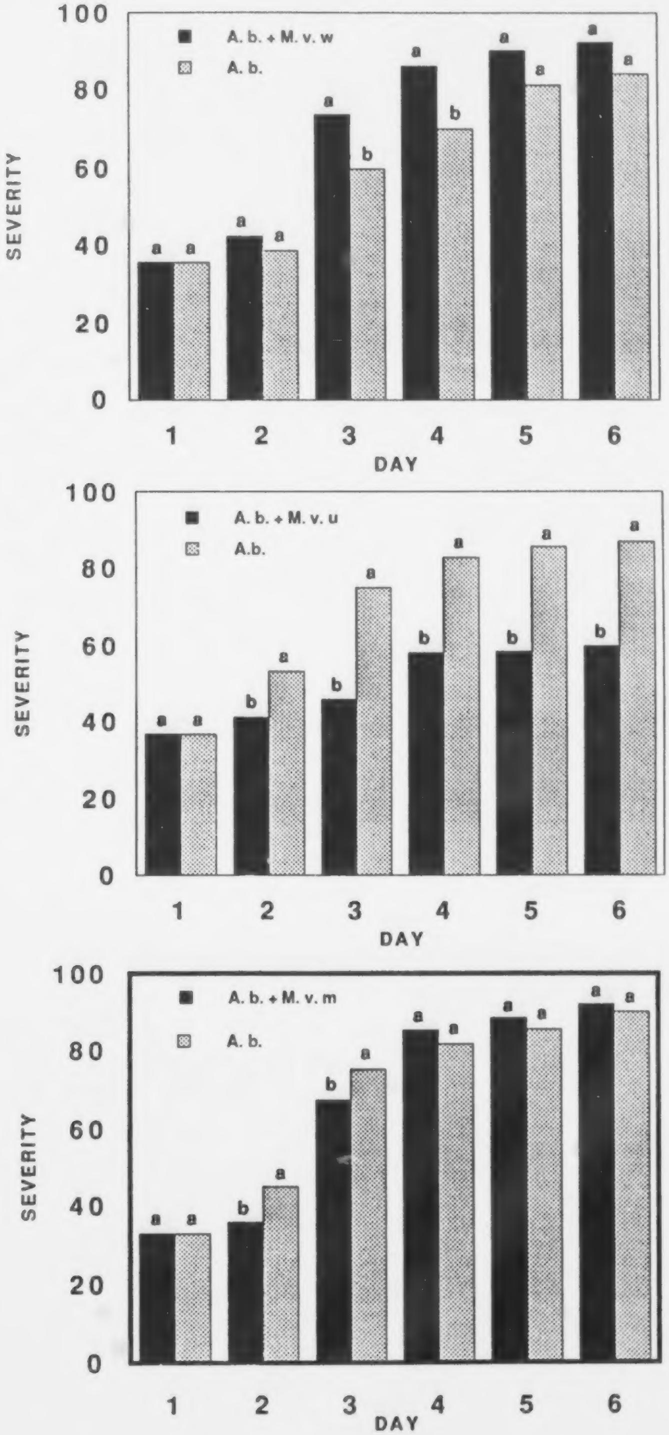


Table 4.1. Days to Appearance of Alternaria Blackspot Symptoms on Excised Leaves of Legend and Reward when *Alternaria brassicae* conidia and Antagonists or Metabolites of *Myrothecium verrucaria* were Applied Simultaneously

Treatment	*Myrothecium verrucaria* Washed	*Myrothecium verrucaria* Unwashed	*Myrothecium verrucaria* Metabolites	*Gliocladium roseum* Isolate 98	*Gliocladium roseum* Isolate 17
A. brassicae + antagonist /metabolites	*3.30 b	5.56 a	3.25 a	4.29 a	2.90 a
	†3.87 a	4.44 a	3.00 a	2.89 a	3.45 a
A. brassicae	*4.28 a	3.16 b	2.96 a	3.92 a	3.21 a
	†3.58 a	2.85 b	2.81 a	2.83 a	3.93 a

The range for the standard error of the mean for values presented in this table is from 0.231 to 0.488.
* refers to results for Legend.
† refers to results for Reward
Vertical comparisons are indicated with a, b.
All comparisons of days to appearance of disease symptoms were made within cultivar within treatments.

Figure 4.5. Severity of lesions on leaves of Legend caused by *Alternaria brassicae* alone and when combined with washed conidia of *Myrothecium verrucaria* (top), unwashed conidia of *M. verrucaria* (middle) and metabolites of *M. verrucaria* (bottom). (A. b. = *Alternaria brassicae*, M. v. = *Myrothecium verrucaria*, w = washed conidia, u = unwashed conidia, m = metabolites of *M. verrucaria*).



■ A. b. + M. v. w
▨ A. b.
SEVERITY
100
80
60
40
20
0
DAY
1
2
3
4
5
6
a a
a a
a b
a b
a a
a a
■ A. b. + M. v. u
▨ A.b.
SEVERITY
100
80
60
40
20
0
DAY
1
2
3
4
5
6
a a
b a
b a
b a
b a
b a
■ A. b. + M. v. m
▨ A. b.
SEVERITY
100
80
60
40
20
0
DAY
1
2
3
4
5
6
a a
b a
b a
a a
a a
a a

Figure 4.6. Lesion severity 5 days post-inoculation on excised leaves of Legend when *Alternaria brassicae* was combined with washed conidia of *Myrothecium verrucaria* (top leaf) and unwashed conidia of *M. verrucaria* (bottom leaf). Fungi were applied simultaneously. The order of droplets for the top leaf from tip to base was *A. brassicae*, sterile distilled water, *A. brassicae* + washed conidia of *M. verrucaria* and washed conidia of *M. verrucaria*. The order of droplets for the bottom leaf from tip to base was *A. brassicae*, sterile distilled water, *A. brassicae* + unwashed conidia of *M. verrucaria* and unwashed conidia of *M. verrucaria*. The droplets were in the same order for both halves of each leaf.

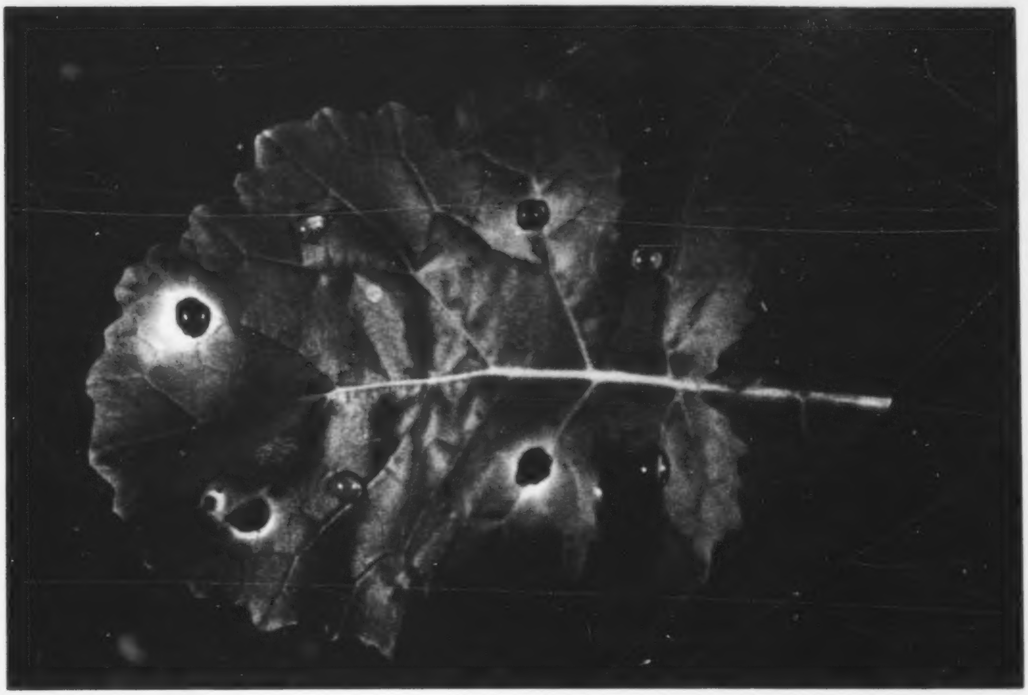


Figure 4.7. Lesion severity 5 days post-inoculation on a leaf of Legend when *Alternaria brassicae* was applied in combination with metabolites of *Myrothecium verrucaria* (from the simultaneous application experiment). The order of droplets from the tip to the base of the leaf was sterile distilled water, *A. brassicae* + metabolites of *M. verrucaria*, metabolites of *M. verrucaria* and *A. brassicae*. The order of the droplets was the same on each half of the leaf.

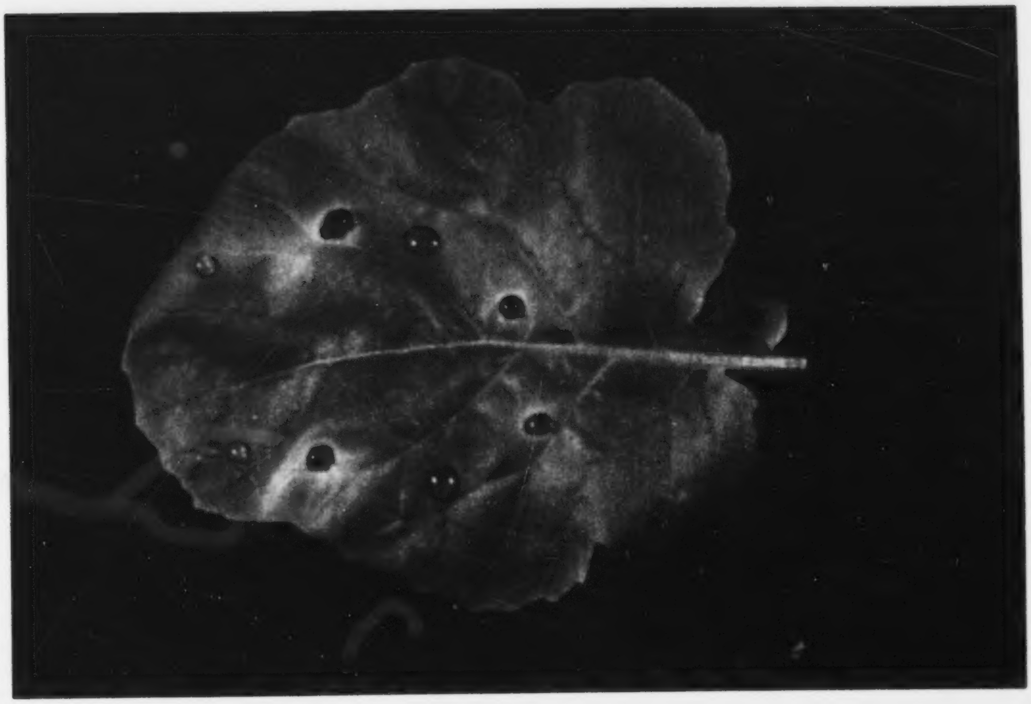
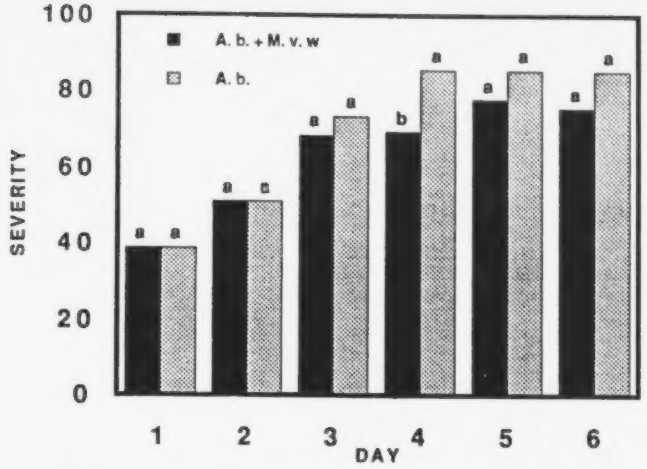
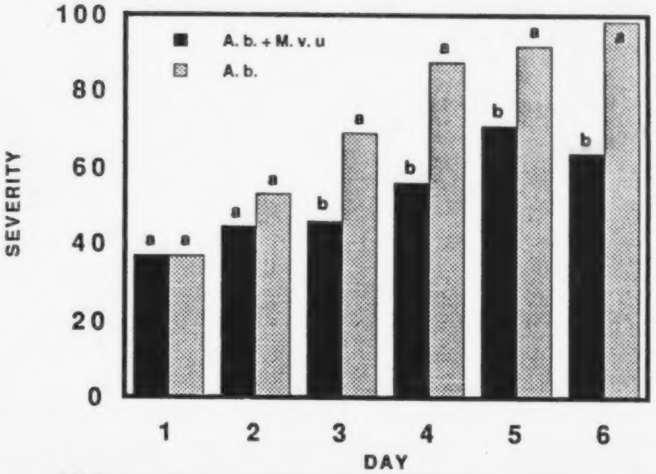


Figure 4.8. Severity of lesions on leaves of Reward caused by *Alternaria brassicae* alone and when combined with washed conidia of *Myrothecium verrucaria* (top), unwashed conidia of *M. verrucaria* (middle) and metabolites of *M. verrucaria* (bottom). (A. b. = *Alternaria brassicae*, M. v. = *Myrothecium verrucaria*, w = washed conidia, u = unwashed conidia, m = metabolites of *M. verrucaria*).


100
80
60
40
20
0
SEVERITY
■ A. b. + M. v. w
▨ A. b.
a a
a a
a a
b a
a a
a a
1
2
3
4
5
6
DAY


100
80
60
40
20
0
SEVERITY
■ A. b. + M. v. u
▨ A. b.
a a
a a
b a
b a
b a
b a
1
2
3
4
5
6
DAY

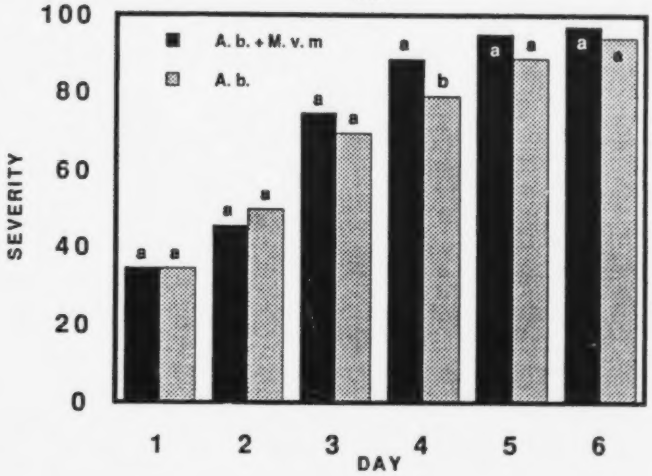

100
80
60
40
20
0
SEVERITY
■ A. b. + M. v. m
▨ A. b.
a a
a a
a a
a b
a a
a a
1
2
3
4
5
6
DAY

Figure 4.9. Lesion severity 5 days post-inoculation on excised leaves of Reward when *Alternaria brassicae* was combined with washed conidia (top leaf) and unwashed conidia (bottom leaf) of *Myrothecium verrucaria*. Fungi were applied simultaneously. The order of droplets on the top leaf from tip to base was *A. brassicae* + unwashed *M. verrucaria*, unwashed *M. verrucaria*, *A. brassicae* and sterile distilled water. The order of droplets for the bottom leaf from tip to base was *A. brassicae*, sterile distilled water, *A. brassicae* + unwashed *M. verrucaria* and unwashed *M. verrucaria* (one droplet of which is missing). The order of the droplets was the same on each half of each leaf.

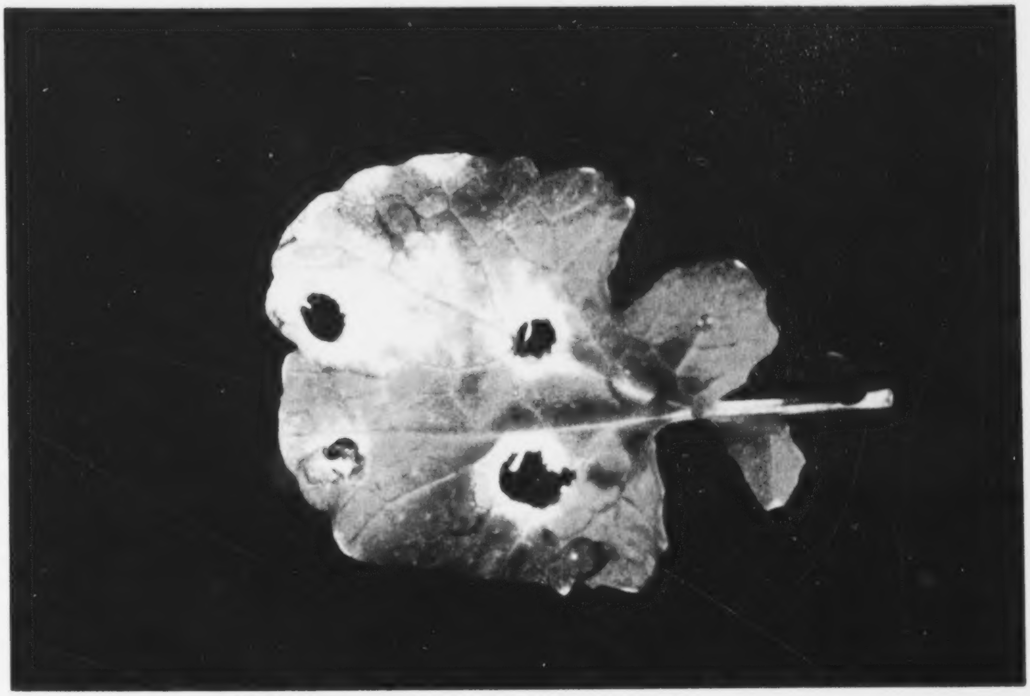
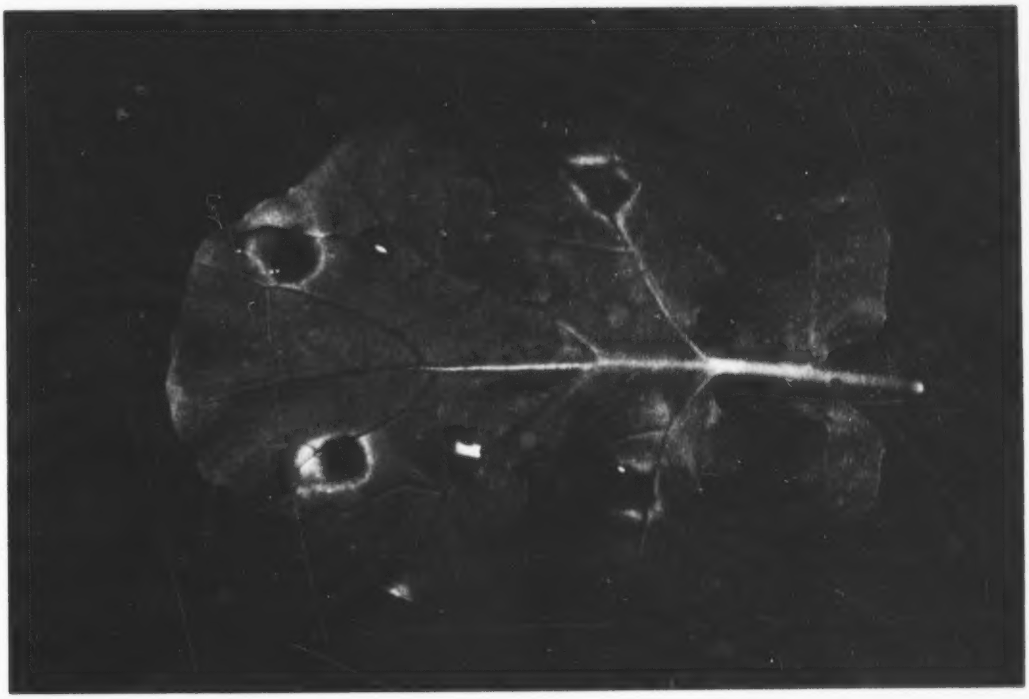


Figure 4.10. Lesion severity 5 days post-inoculation on a leaf of Reward when *Alternaria brassicae* was combined with metabolites of *Myrothecium verrucaria* (from the simultaneous application experiment). The order of the droplets from tip to base was *A. brassicae*, sterile distilled water, *A. brassicae* + metabolites of *M. verrucaria* and metabolites of *M. verrucaria*. The droplets were in the same order on each half of the leaf.



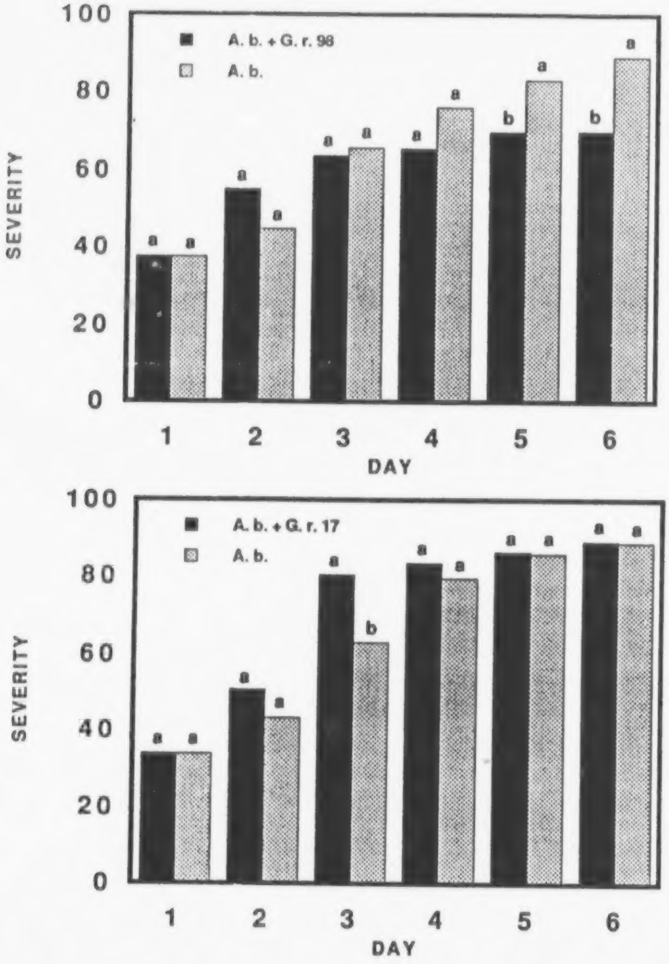
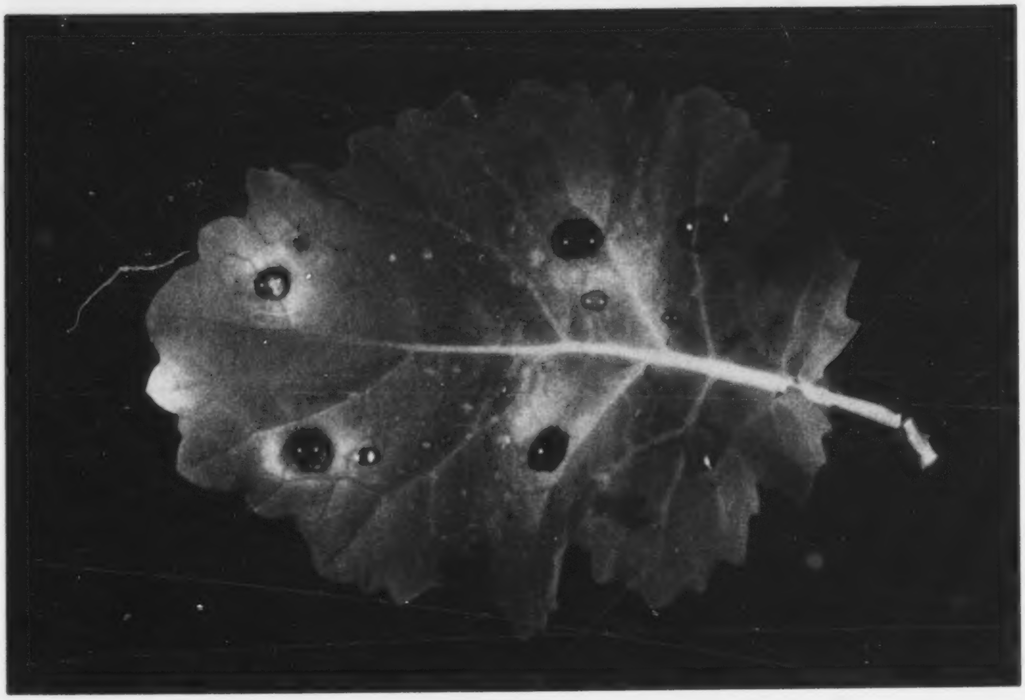
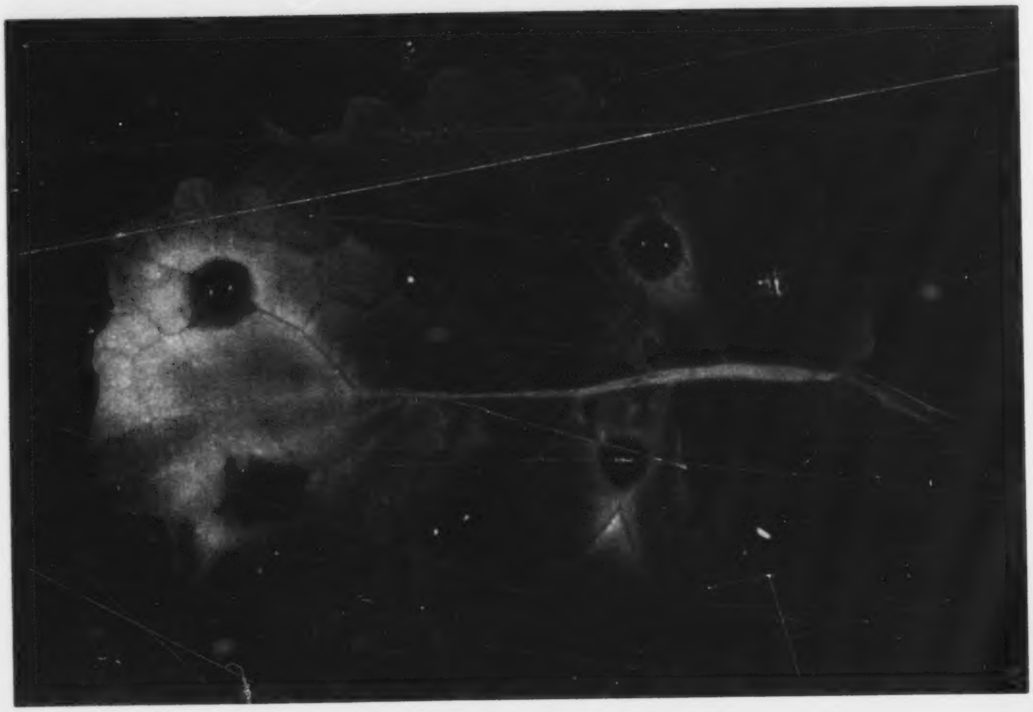


Figure 4.11. Severity of lesions on leaves of Legend caused by *Alternaria brassicae* alone and when applied in combination with *Gliocladium roseum* isolate 98 (top) and *G. roseum* isolate 17 (bottom). (A. b. = *Alternaria brassicae*, G. r. = *Gliocladium roseum*, 17 and 98 refer to the isolates of *G. roseum*).

Figure 4.12. Lesion severity 5 days post-inoculation on leaves of Legend when *Alternaria brassicae* was applied in combination with *Gliocladium roseum* isolate 98 (top leaf) and isolate 17 (bottom leaf) when fungi were applied simultaneously. The order of droplets for the top leaf was *A. brassicae* + *G. roseum* isolate 98, *G. roseum* isolate 98, *A. brassicae* and sterile distilled water. The order of droplets for the bottom leaf was *A. brassicae*, sterile distilled water, *A. brassicae* + *G. roseum* isolate 17 and *G. roseum* isolate 17. The order of droplets was the same on each half of each leaf.





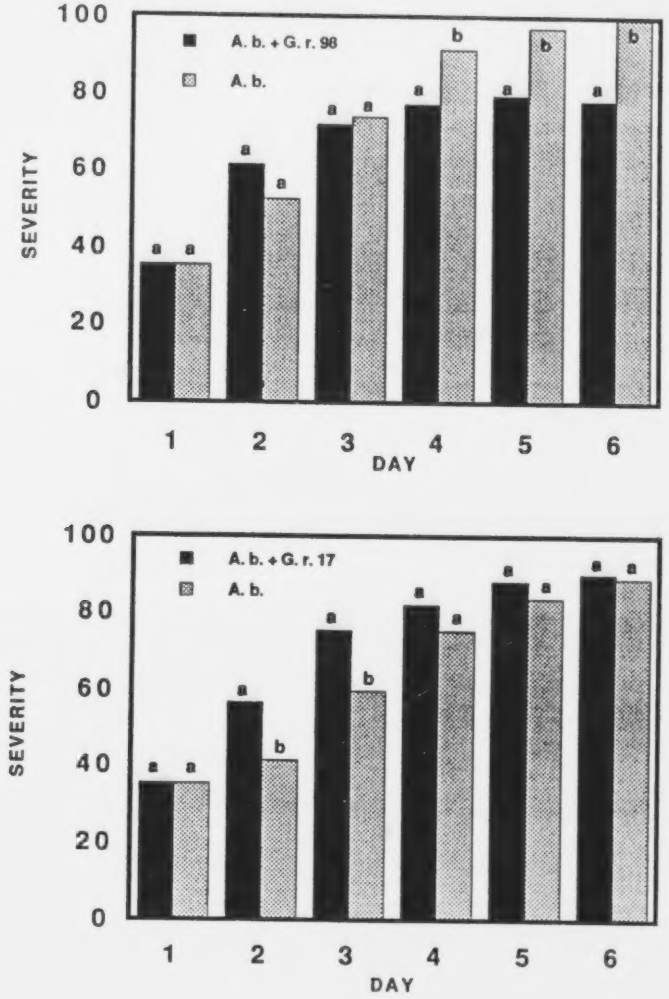
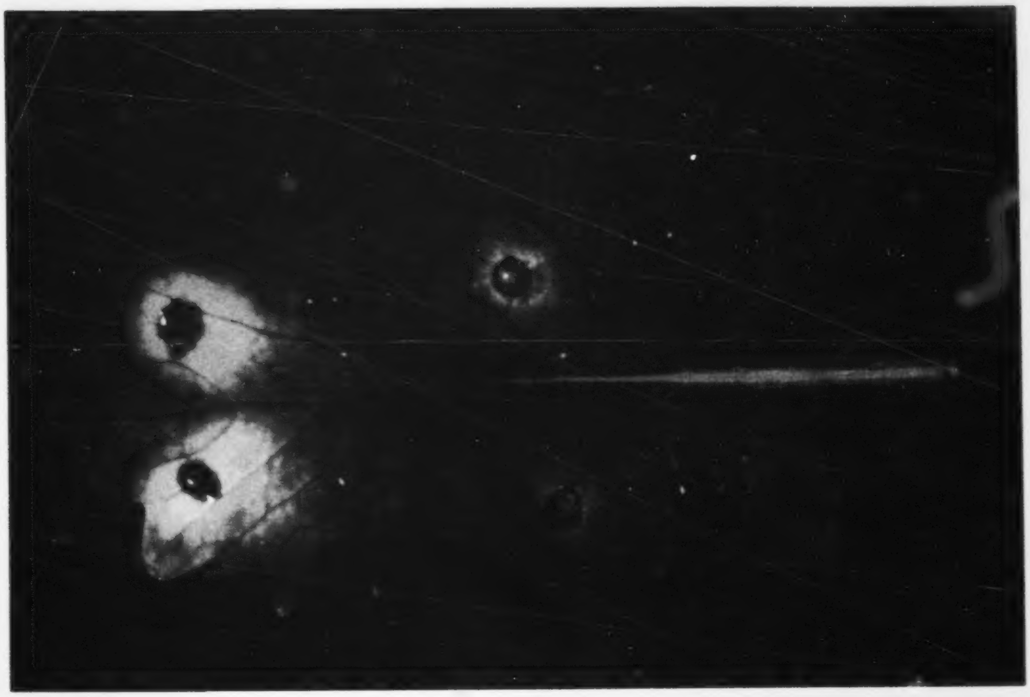


Figure 4.13. Severity of lesions on leaves of Reward caused by *Alternaria brassicae* alone and when applied in combination with *Gliocladium roseum* isolate 98 (top) and *G. roseum* isolate 17 (bottom). (A. b. = *Alternaria brassicae*, G. r. = *Gliocladium roseum*, 17 and 98 refer to the isolates of *G. roseum*).

Figure 4.14. Lesion severity 5 days post-inoculation on leaves of Reward when *Alternaria brassicae* was applied in combination with *Gliocladium roseum* isolate 98 (top leaf) and isolate 17 (bottom leaf) when fungi were applied simultaneously. The order of droplets from tip to base for the top leaf was *A. brassicae*, sterile distilled water, *A. brassicae* + *G. roseum* isolate 98 and lastly *G. roseum* isolate 98. The order of droplets from tip to base for the bottom leaf was *A. brassicae*, sterile distilled water, *A. brassicae* + *G. roseum* isolate 17 and G. *roseum* isolate 17. The droplets were in the same order on each half of each leaf.



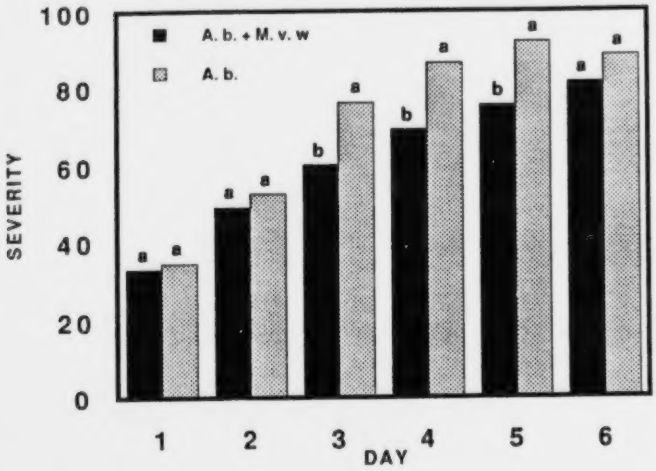
B

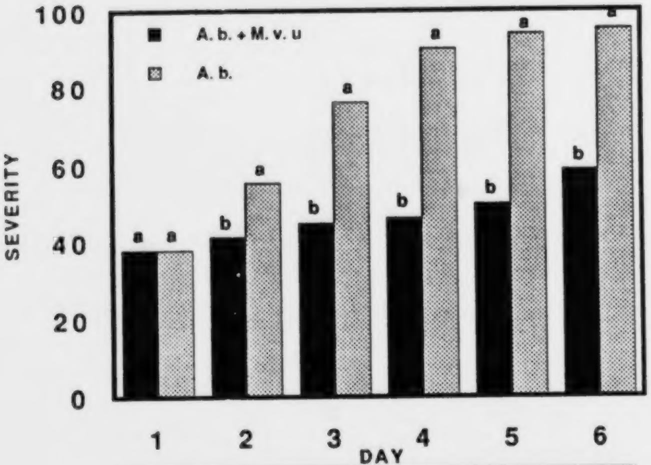
Table 4.2. Days to Appearance of Alternaria Blackspot Symptoms on Excised Leaves of Legend and Reward when *Alternaria brassicae* conidia were Applied 10 hours before the Antagonists and Metabolites of *Myrothecium verrucaria*

Treatment	*Myrothecium verrucaria* Washed	*Myrothecium verrucaria* Unwashed	*Myrothecium verrucaria* Metabolites	*Gliocladium roseum* Isolate 98	*Gliocladium roseum* Isolate 17
A. brassicae + antagonist /metabolites	*3.37 a	5.64 a	2.54 a	2.25 a	2.50 a
	†3.29 a	4.08 a	2.38 a	2.53 a	2.83 a
A. brassicae	*2.81 a	3.00 b	2.99 a	2.63 a	2.67 a
	†2.75 a	2.42 b	2.55 a	2.63 a	2.91 a

The range for the standard error of the mean for values presented in this table is from 0.021 to 0.488.
* indicates results for Legend.
† indicates results for Reward.
Vertical comparisons are indicated with a, b.
All comparisons of days to appearance of disease symptoms were made within cultivar within treatments

Figure 4.15. Severity of lesions on leaves of Legend caused by *Alternaria brassicae* alone and when washed conidia of *Myrothecium verrucaria* (top), unwashed conidia of *M. verrucaria* (middle) and metabolites of *M. verrucaria* (bottom) were applied ten hours after *A. brassicae*. (A. b. = *A. brassicae*, M. v. = *M. verrucaria*, w = washed conidia, u = unwashed conidia, m = metabolites of *M. verrucaria*).


■ A. b. + M. v. w
▨ A. b.
SEVERITY
0
20
40
60
80
100
DAY
1
2
3
4
5
6
a a
a a
b a
b a
b a
a a


■ A. b. + M. v. u
▨ A. b.
SEVERITY
0
20
40
60
80
100
DAY
1
2
3
4
5
6
a a
b a
b a
b a
b a
b a

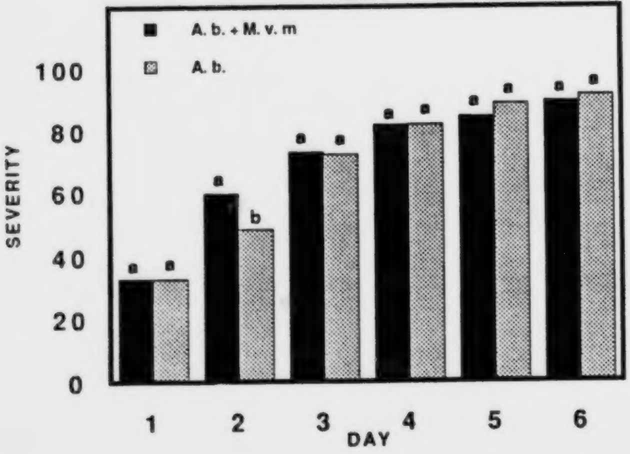

■ A. b. + M. v. m
▨ A. b.
SEVERITY
0
20
40
60
80
100
DAY
1
2
3
4
5
6
a a
a b
a a
a a
a a
a a

Figure 4.16. Lesion severity 5 days post-inoculation on excised leaves of Legend when *Alternaria brassicae* was applied in combination with washed conidia of *M. verrucaria* (top leaf) and unwashed conidia of *M. verrucaria* (bottom leaf). *Alternaria brassicae* was applied prior to *M. verrucaria*. The order of droplets for the top leaf from tip to base was sterile distilled water, *A. brassicae* + washed conidia of *M. verrucaria*, washed conidia of *M. verrucaria* and *A. brassicae*. The order of droplets for the bottom leaf from tip to base was unwashed conidia of *M. verrucaria*, *A. brassicae*, sterile distilled water and *A. brassicae* + unwashed conidia of *M. verrucaria*. The order of the droplets was the same on each half of each leaf.

Figure 4.17. Lesion severity 5 days post-inoculation on a leaf of Legend when *Alternaria brassicae* was applied in combination with metabolites of *Myrothecium verrucaria*. *Alternaria brassicae* was applied prior to metabolites of *M. verrucaria* . The order of droplets on the leaf was metabolites of *M. verrucaria*, *A. brassicae*, sterile distilled water and *A. brassicae* + metabolites of *M. verrucaria*. The order of the droplets was the same on each half of the leaf.

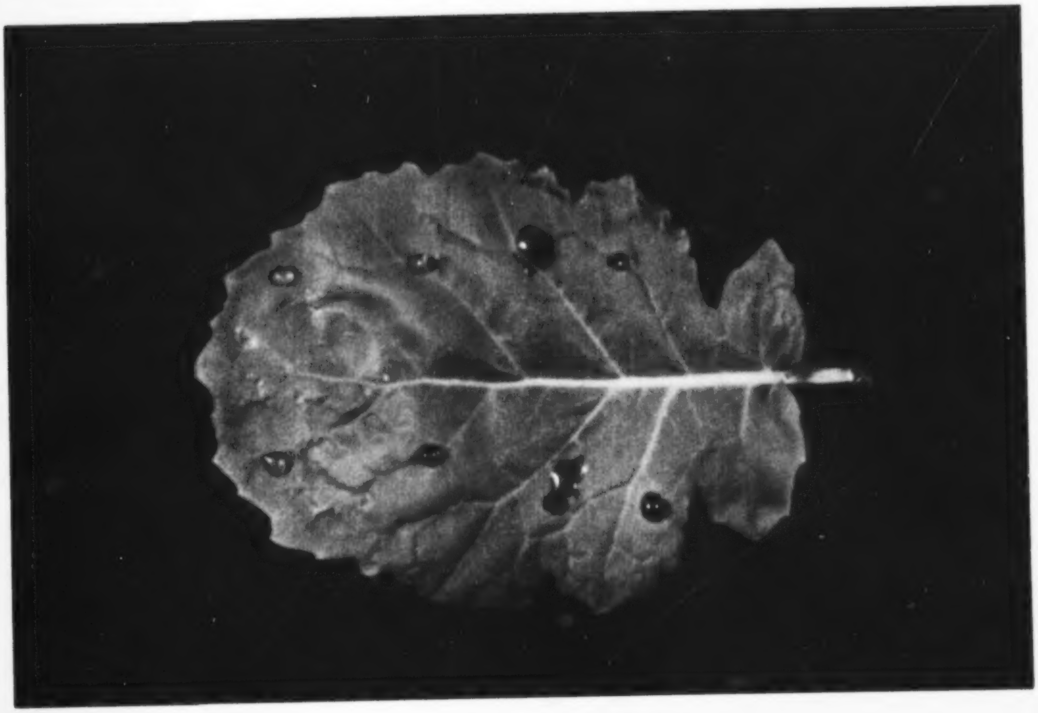
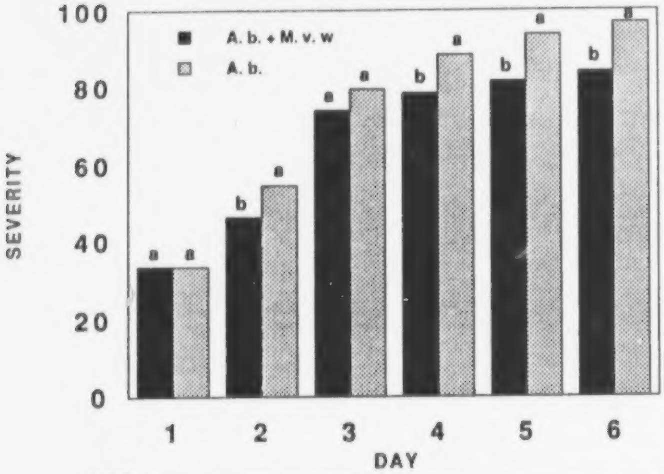
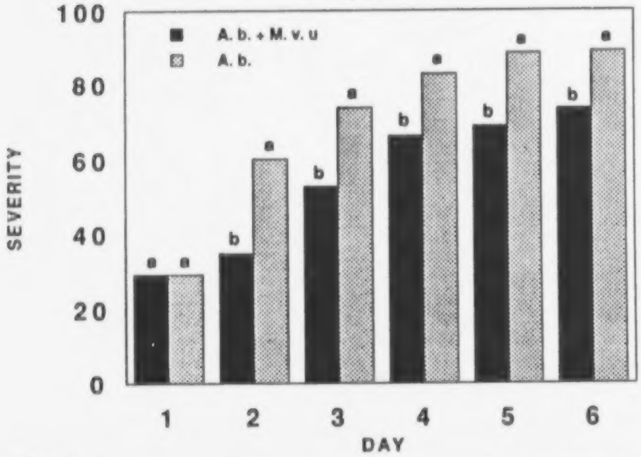


Figure 4.18. Severity of lesions on leaves of Reward caused by *Alternaria brassicae* alone and when washed conidia of *Myrothecium verrucaria* (top), unwashed conidia of *M. verrucaria* (middle) and metabolites of *M. verrucaria* (bottom) were applied ten hours after *A. brassicae.* (A. b. = *A. brassicae*, M. v. = *M. verrucaria*, w = washed conidia, u = unwashed conidia, m = metabolites of *M. verrucaria*)


■ A. b. + M. v. w
▨ A. b.
SEVERITY
100
80
60
40
20
0
DAY
1
2
3
4
5
6
a a
b a
a a
b a
b a
b a


■ A. b. + M. v. u
▨ A. b.
SEVERITY
100
80
60
40
20
0
DAY
1
2
3
4
5
6
a a
b a
b a
b a
b a
b a

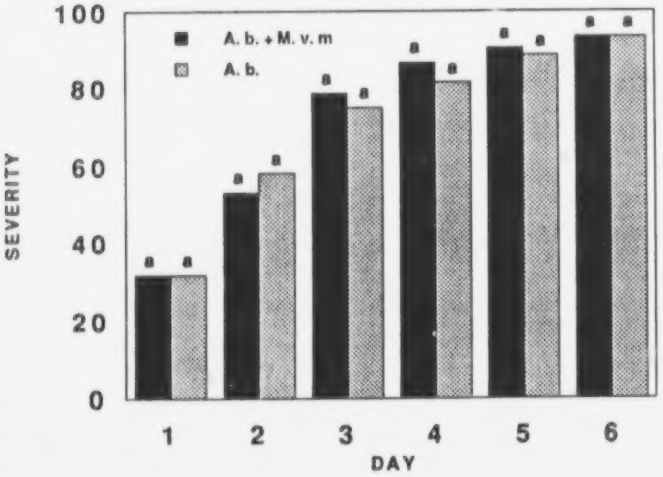

■ A. b. + M. v. m
▨ A. b.
SEVERITY
100
80
60
40
20
0
DAY
1
2
3
4
5
6
a a
a a
a a
a a
a a
a a

Figure 4.19. Lesion severity 5 days post-inoculation on excised leaves of Reward when *Alternaria brassicae* was applied in combination with washed conidia of *M. verrucaria* (top leaf) and unwashed conidia of *M. verrucaria* (bottom leaf). *Alternaria brassicae* was applied 10 hours prior to *M. verrucaria*. The order of droplets for the top leaf from tip to base was washed conidia of *M. verrucaria*, *A. brassicae*, sterile distilled water and *A. brassicae* + washed conidia of *M. verrucaria*. The order of droplets for the bottom leaf was sterile distilled water, *A. brassicae* + unwashed conidia of *M. verrucaria*, unwashed conidia of *M. verrucaria* and *A. brassicae*. The droplets were in the same order on each half of each leaf. Note that one droplet of sterile distilled water on the bottom leaf was contaminated and slight chlorosis was apparent.

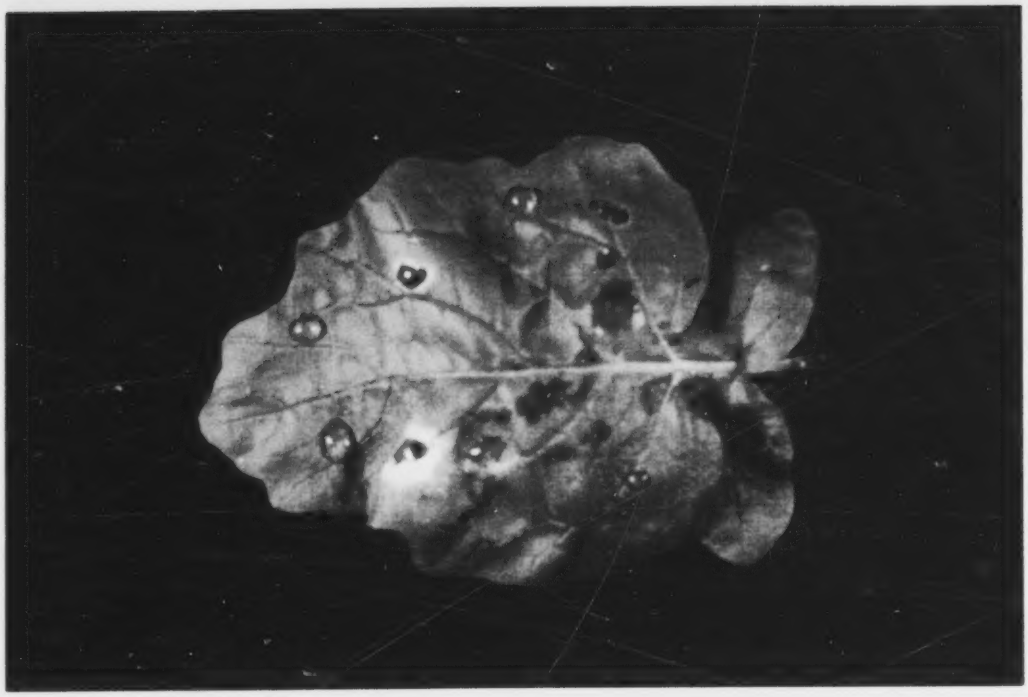
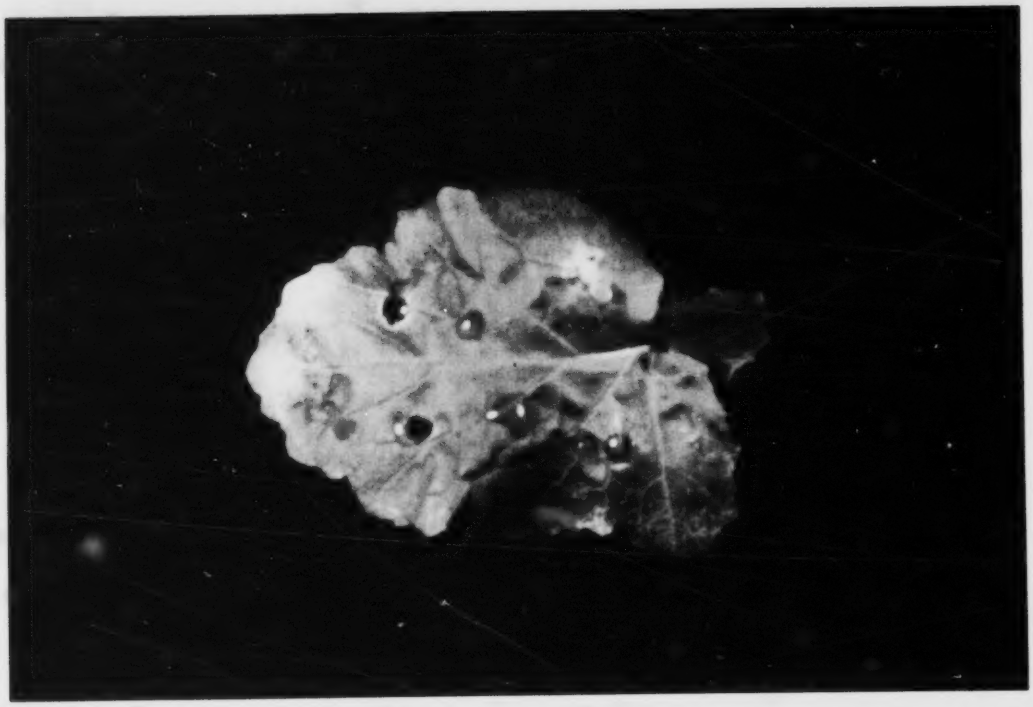


Figure 4.20. Lesion severity 5 days post-inoculation on a leaf of Reward when *Alternaria brassicae* was applied in combination with metabolites of *Myrothecium verrucaria*. *Alternaria brassicae* was applied 10 hours prior to metabolites of *M. verrucaria*. The order of droplets from the tip to the base of the leaf was sterile distilled water, *A. brassicae* + metabolites of *M. verrucaria*, metabolites of *M. verrucaria* and *A. brassicae*. The droplets were in the same order on each half of the leaf.



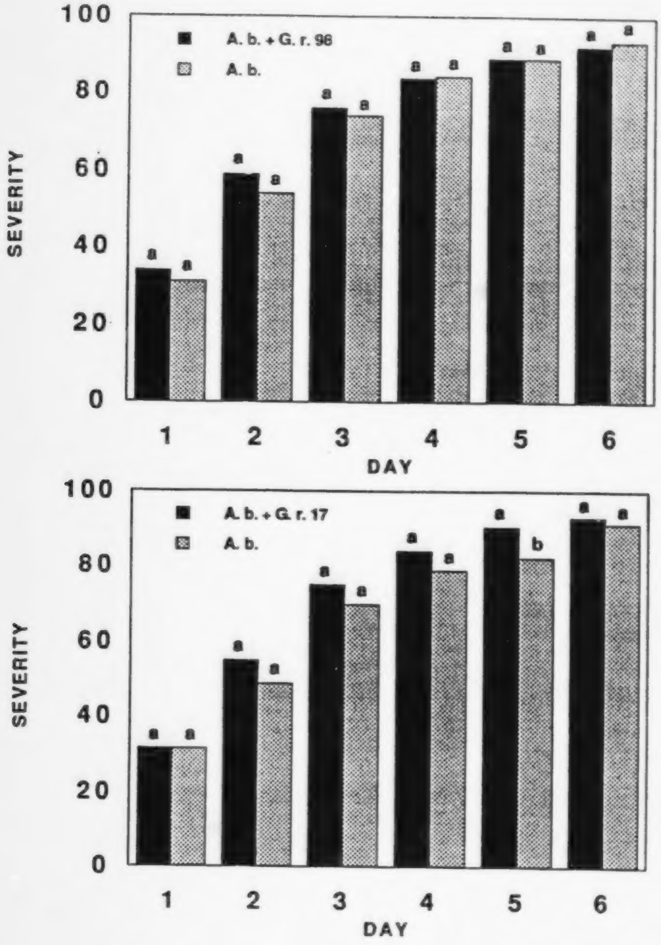
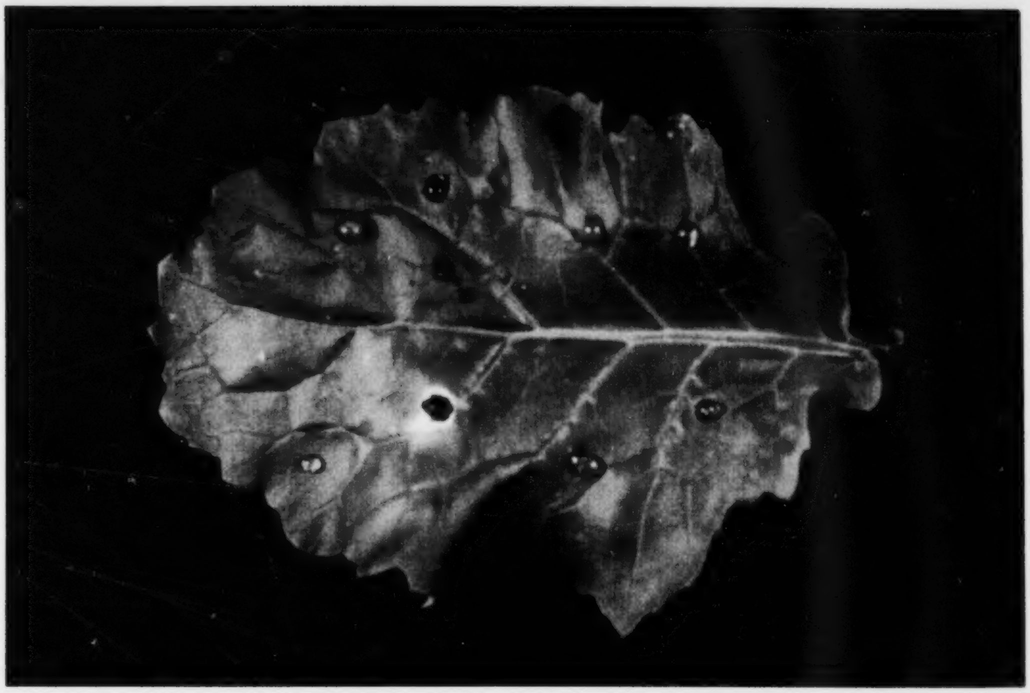
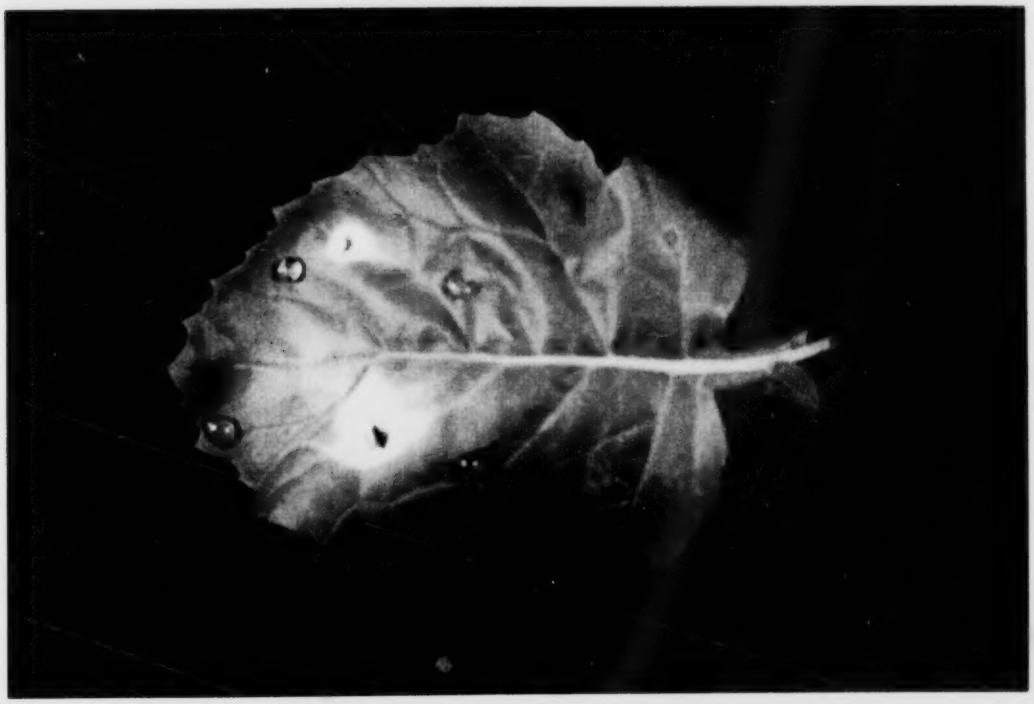


Figure 4.21. Severity of lesions on leaves of Legend caused by *Alternaria brassicae* alone and when conidia of *Gliocladium roseum* isolate 98 (top) and *G. roseum* isolate 17 (bottom) were applied ten hours after *A. brassicae.* (A. b. = *Alternaria brassicae*, G. r. = *Gliocladium roseum*, 17 and 98 refer to the isolates of *G. roseum*).

Figure 4.22. Lesion severity 5 days post-inoculation on a leaves of Legend when *Alternaria brassicae* was combined with *Gliocladium roseum* isolate 98 (top leaf) and *G. roseum* isolate 17 (bottom leaf) when *A. brassicae* was applied prior to *G. roseum.* The order of droplets of the top leaf from the tip to the base was sterile distilled water, *A. brassicae* + *G. roseum* isolate 98, *G. roseum* isolate 98 and *A. brassicae.* The order of droplets of the bottom leaf from the tip to the base was sterile distilled water, *A. brassicae* + *G. roseum* isolate 17, *G. roseum* isolate 17 and *A. brassicae.* The order of the droplets was the same on each half of each leaf.





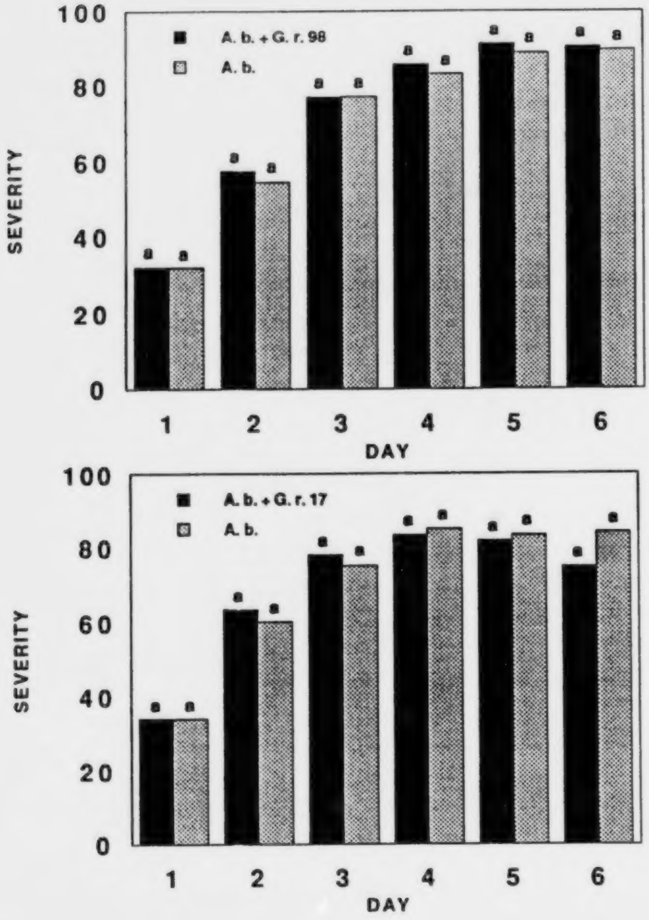


Figure 4.23. Severity of lesions on leaves of Reward caused by *Alternaria brassicae* alone and when conidia of *Gliocladium roseum* isolate 98 (top) and *G. roseum* isolate 17 (bottom) were applied ten hours after *A. brassicae.* (A. b. = *Alternaria brassicae*, G. r. = *Gliocladium roseum*, 17 and 98 refer to the isolates of *G. roseum*).

Figure 4.24. Lesion severity 5 days post inoculation on leaves of Reward when *Alternaria brassicae* was combined with *Gliocladium roseum* isolate 98 (top leaf) or isolate 17 (bottom leaf). *Alternaria brassicae* was applied prior to *G. roseum.* The order of droplets for the top leaf from tip to base was *G. roseum* isolate 98, *A. brassicae*, sterile distilled water and *A. brassicae* + *G. roseum* isolate 98. The order of droplets for the bottom leaf from tip to base was *G. roseum* isolate 17, *A. brassicae*, sterile distilled water and *A. brassicae* + *G. roseum* isolate 17. The droplets were in the same order for both halves of each leaf.

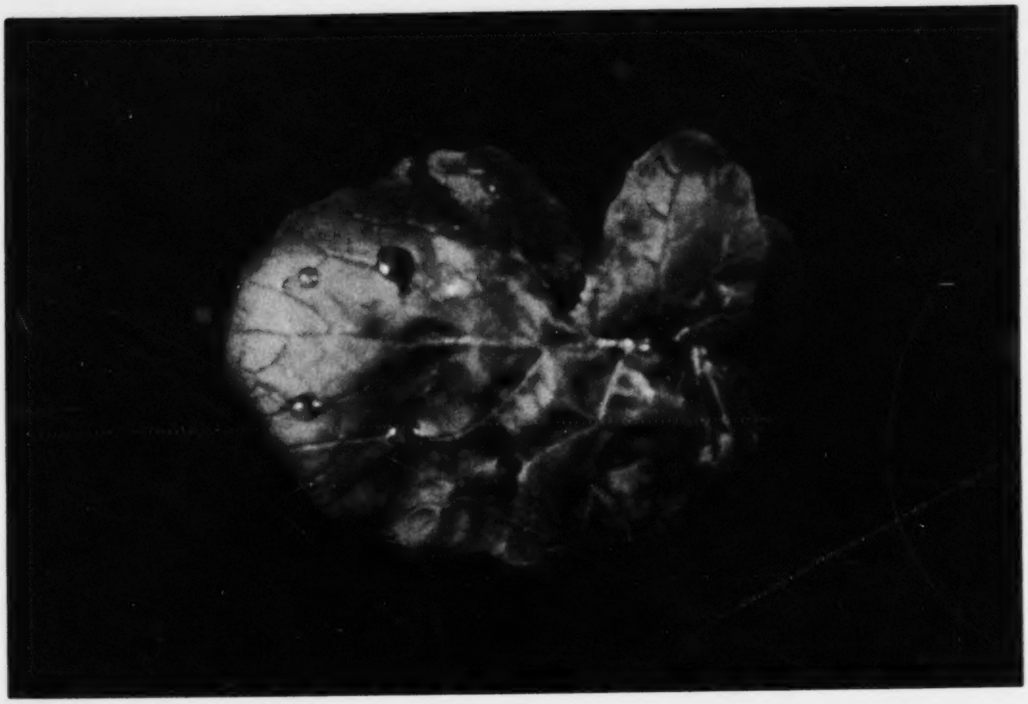
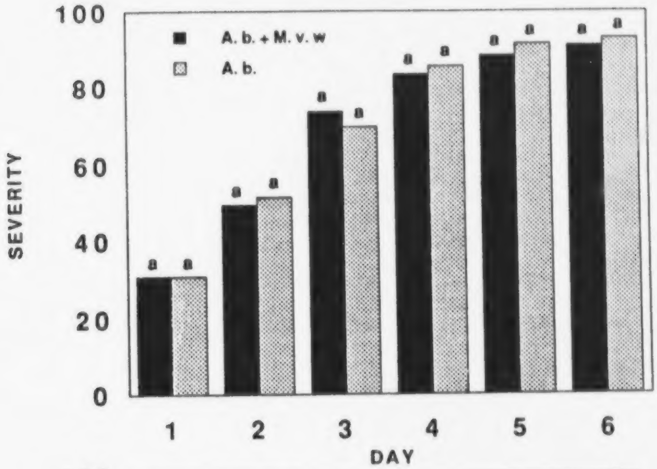


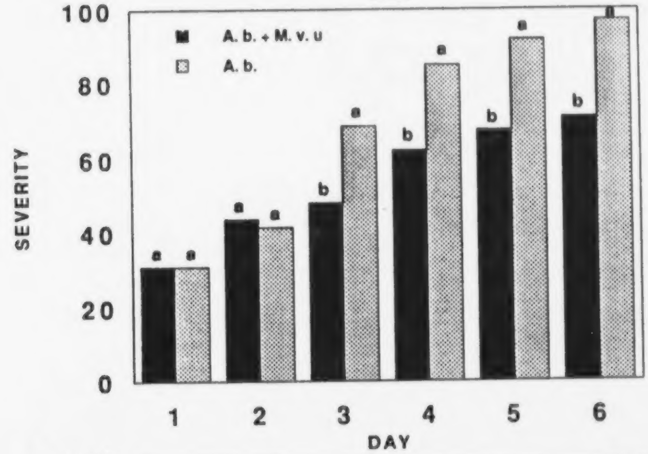
Table 4.3. Days to Appearance of Alternaria Blackspot Symptoms on Excised Leaves of Legend and Reward when Antagonists and Metabolites of *Myrothecium verrucaria* were Applied 10 hours before *Alternaria brassicae* conidia

Treatment	*Myrothecium verrucaria* Washed	*Myrothecium verrucaria* Unwashed	*Myrothecium verrucaria* Metabolites	*Gliocladium roseum* Isolate 98	*Gliocladium roseum* Isolate 17
A. brassicae + antagonist /metabolites	*2.82 a †---	4.44 a ---	3.08 a 2.43 a	2.71 a 2.20 a	4.04 a 2.49 a
A. brassicae	*2.69 a †2.17	3.21 b 2.20	3.04 a 2.44 a	3.21 a 2.20 a	3.13 b 2.19 a

The range for the standard error of the mean for values presented in this table is from 0.115 to 0.273.
* refers to results for Legend.
† refers to results for Reward.
Vertical comparisons are indicated with a, b.
All comparisons of days to appearance of disease symptoms were made within cultivar within treatments.
Results for Alternaria Blackspot development which were skewed by the presence of disease caused by *M. verrucaria* are not presented in this table.

Figure 4.25. Severity of lesions on leaves of Legend caused by *Alternaria brassicae* alone and when washed conidia of *Myrothecium verrucaria* (top), unwashed conidia of *M. verrucaria* (middle) and metabolites of *M. verrucaria* (bottom) were applied ten hours prior *A. brassicae*. (A. b. = *A. brassicae*, M. v. = *M. verrucaria*, w = washed conidia of *M. verrucaria*, u = unwashed conidia of *M. verrucaria*, m = metabolites of *M. verrucaria*).


■ A. b. + M. v. w
▨ A. b.
SEVERITY
0
20
40
60
80
100
a a
a a
a a
a a
a a
a a
1
2
3
4
5
6
DAY


■ A. b. + M. v. u
▨ A. b.
SEVERITY
0
20
40
60
80
100
a a
a a
b a
b a
b a
b a
1
2
3
4
5
6
DAY

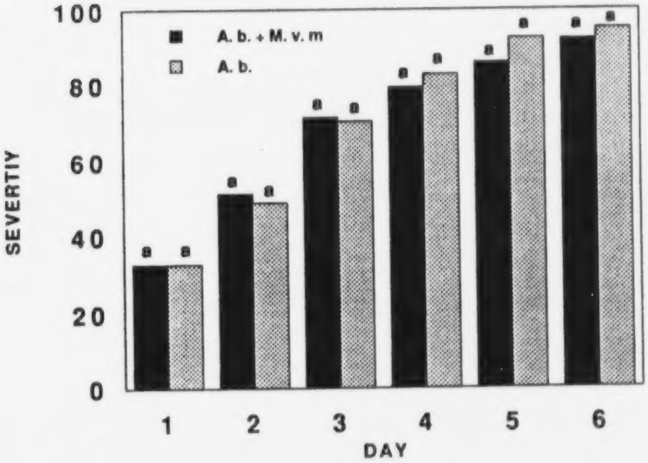
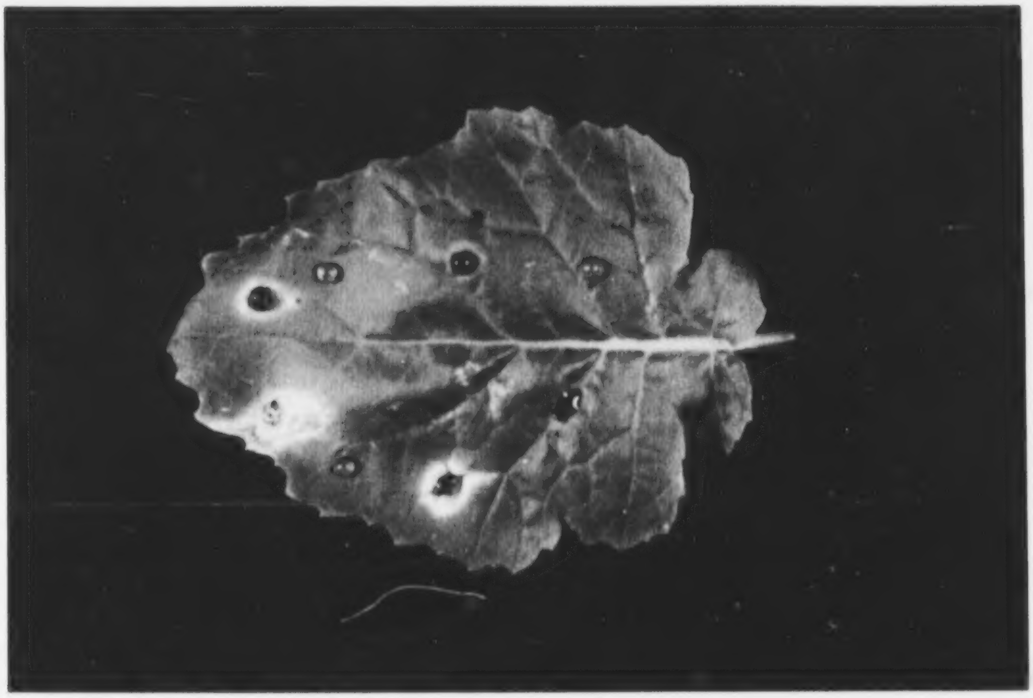

■ A. b. + M. v. m
▨ A. b.
SEVERTIY
0
20
40
60
80
100
a a
a a
a a
a a
a a
a a
1
2
3
4
5
6
DAY

Figure 4.26. Lesion severity 5 days post-inoculation on excised leaves of Legend when *Alternaria brassicae* was in combination with washed conidia of *M. verrucaria* (top leaf) and unwashed conidia of *M. verrucaria* (bottom leaf). *Myrothecium verrucaria* was applied 10 hours prior to *A. brassicae.* The order of droplets from tip to base of the top leaf was *A. brassicae* + washed *M. verrucaria* conidia, washed conidia of *M. verrucaria*, *A. brassicae* and sterile distilled water. The order of droplets on the bottom leaf from tip to base was sterile distilled water, *A. brassicae* + unwashed conidia of *M. verrucaria*, unwashed conidia of *M. verrucaria* and *A. brassicae.* The droplets were in the same order on each half of each leaf.



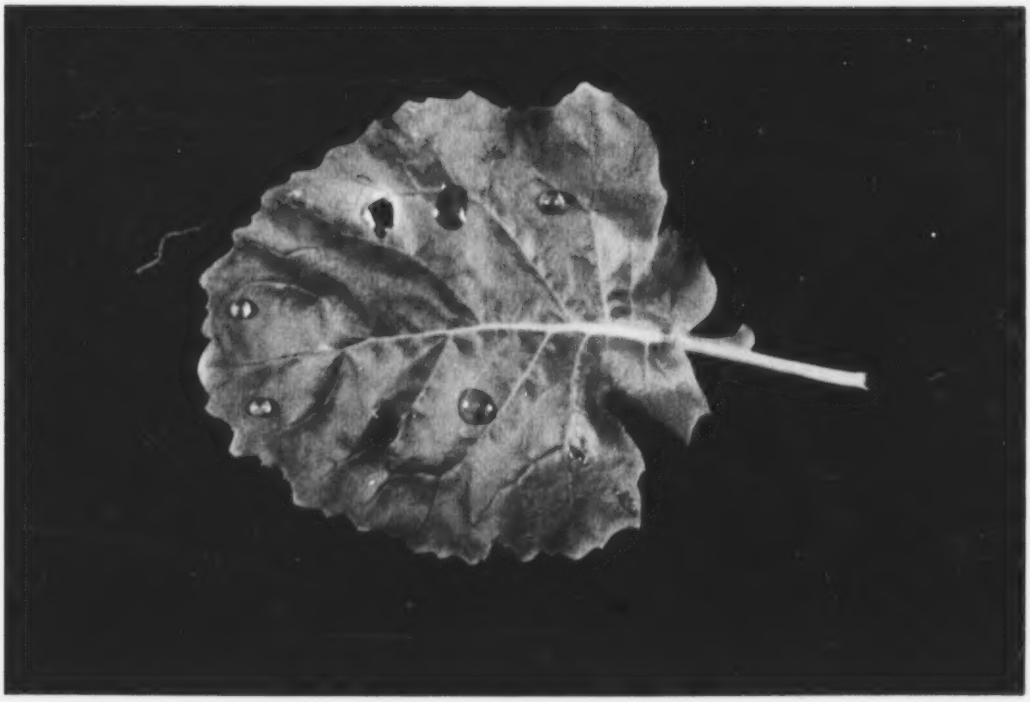


Figure 4.27. Lesion severity 5 days post-inoculation on a leaf of Legend when *Alternaria brassicae* was combined with metabolites *Myrothecium verrucaria* which were applied 10 hours prior to the conidia of *A. brassicae*. The order of droplets from the tip to the base of the leaf was metabolites of *M. verrucaria*, *A. brassicae*, sterile distilled water and *A. brassicae* + metabolites of *M. verrucaria*. The droplets were in the same order on each half of the leaf.

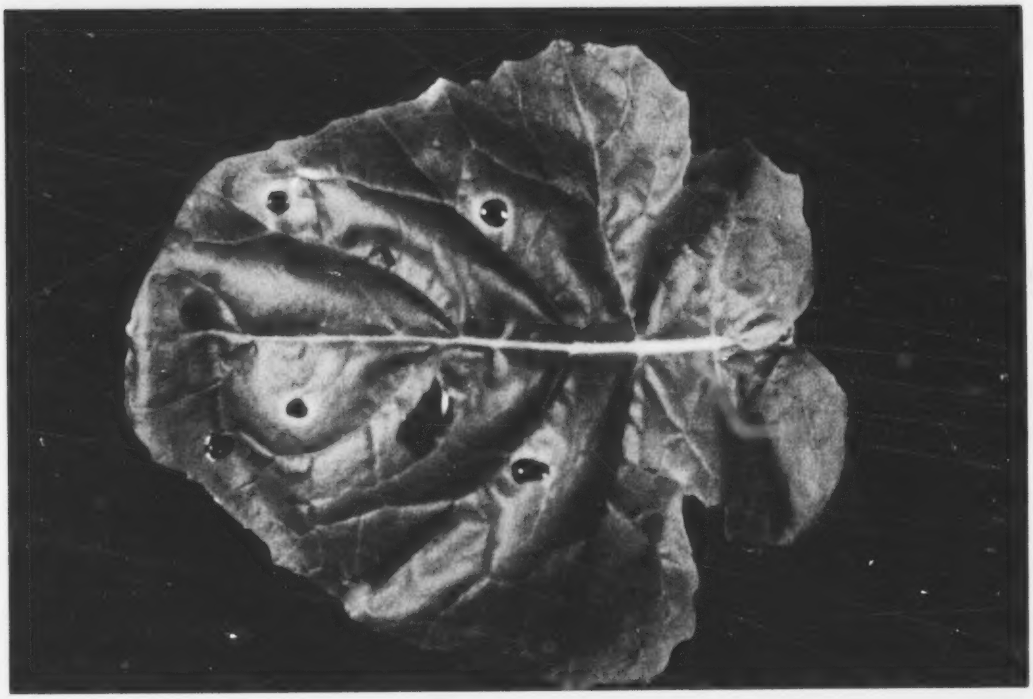
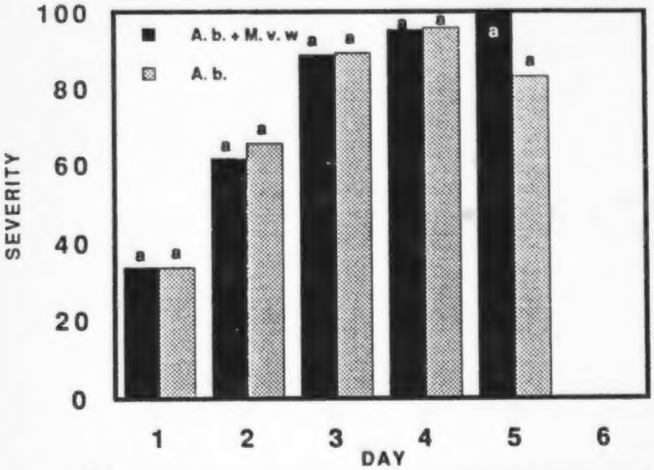
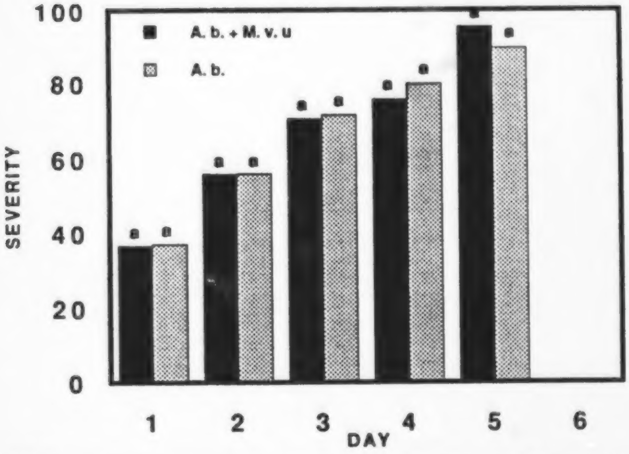


Figure 4.28. Severity of lesions on leaves of Reward caused by *Alternaria brassicae* alone and when washed conidia of *Myrothecium verrucaria* (top), unwashed conidia of *M. verrucaria* (middle) and metabolites of *M. verrucaria* (bottom) were applied ten hours prior *A. brassicae.* (A. b. = *Alternaria brassicae,* M. v. = *Myrothecium verrucaria*, w = washed conidia of *M. verrucaria*, u = unwashed conidia of *M. verrucaria*, m = metabolites of *M. verrucaria*).


A. b. + M. v. w
A. b.
SEVERITY
0
20
40
60
80
100
a
1
2
3
4
5
6
DAY


A. b. + M. v. u
A. b.
SEVERITY
0
20
40
60
80
100
a
1
2
3
4
5
6
DAY

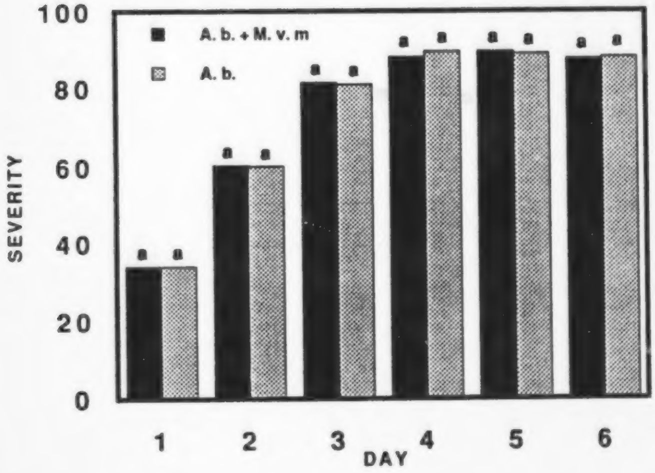
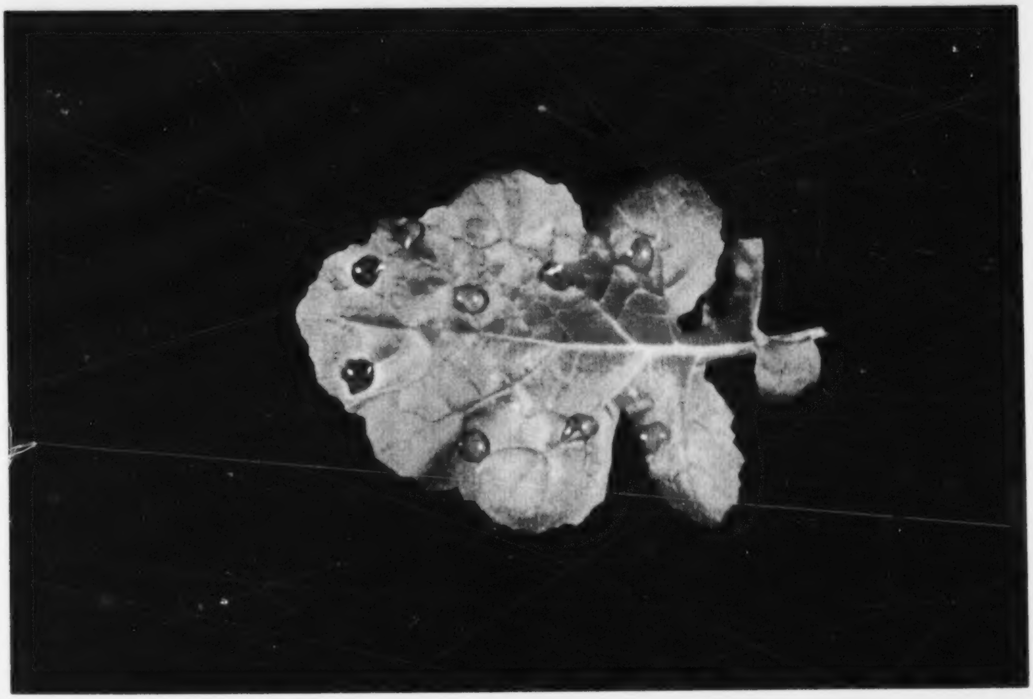

A. b. + M. v. m
A. b.
SEVERITY
0
20
40
60
80
100
a
1
2
3
4
5
6
DAY

Figure 4.29. Lesion severity 5 days post-inoculation on excised leaves of Reward when *Alternaria brassicae* was treated with washed conidia of *M. verrucaria* (top leaf) and unwashed conidia of *M. verrucaria* (bottom leaf). *Myrothecium verrucaria* was applied prior to *A. brassicae.* The order of droplets for the top leaf from tip to base was *A. brassicae*, sterile distilled water, *A. brassicae* + washed conidia of *M. verrucaria* and washed conidia of *M. verrucaria.* The order of droplets for the bottom leaf from tip to base was *A. brassicae*, sterile distilled water, *A. brassicae* + unwashed conidia of *M. verrucaria* and unwashed conidia of *M. verrucaria.* The order of the droplets was the same on each half of each leaf. Note the presence of disease caused by *M. verrucaria* where unwashed conidia were applied to the leaf.



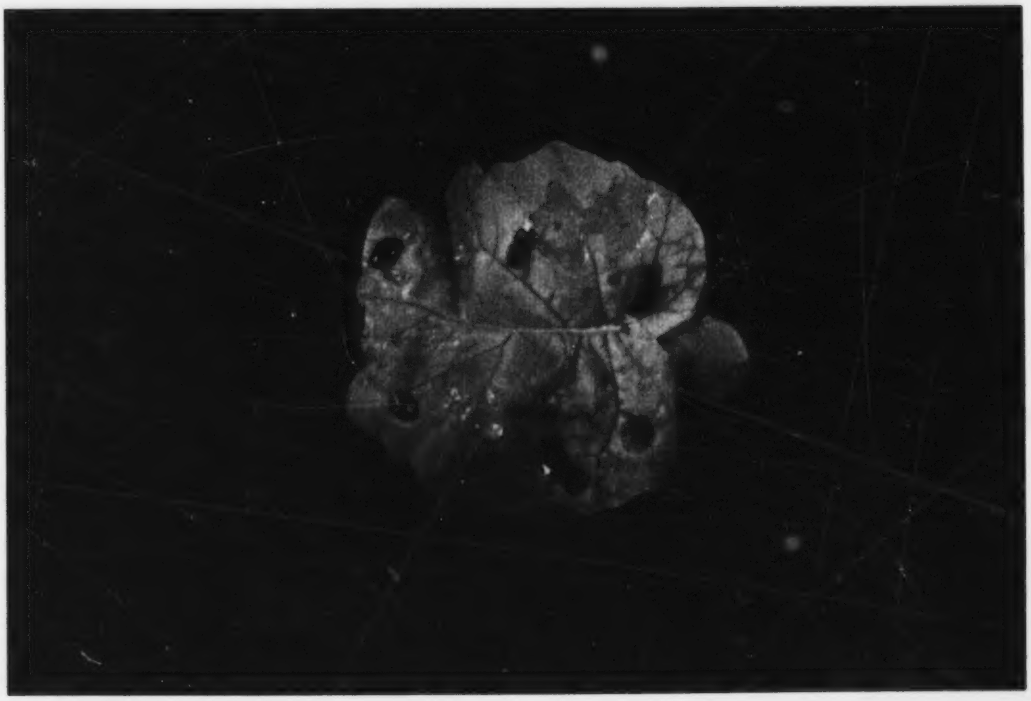
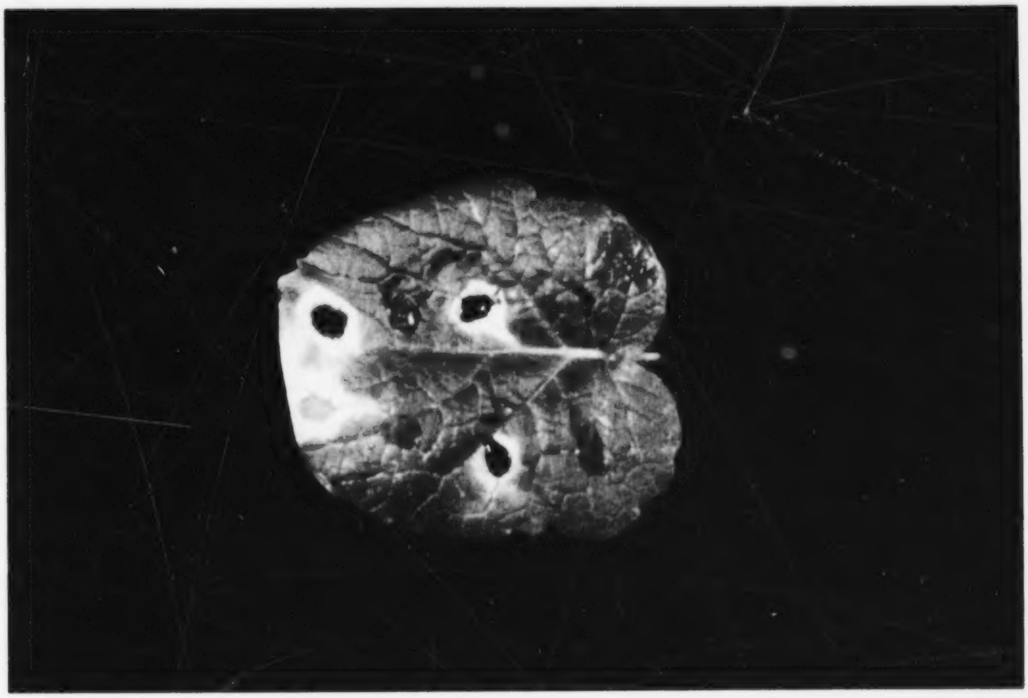


Figure 4.30. Lesion severity 5 days post-inoculation on a leaf of Reward when *Alternaria brassicae* was combined with metabolites of *Myrothecium verrucaria* which were applied prior to *A. brassicae.* The order of droplets from the tip to the base of the leaf was *A. brassicae* + metabolites of *M. verrucaria*, metabolites of *M. verrucaria*, *A. brassicae* and sterile distilled water. The droplets were in the same order on each half of the leaf.



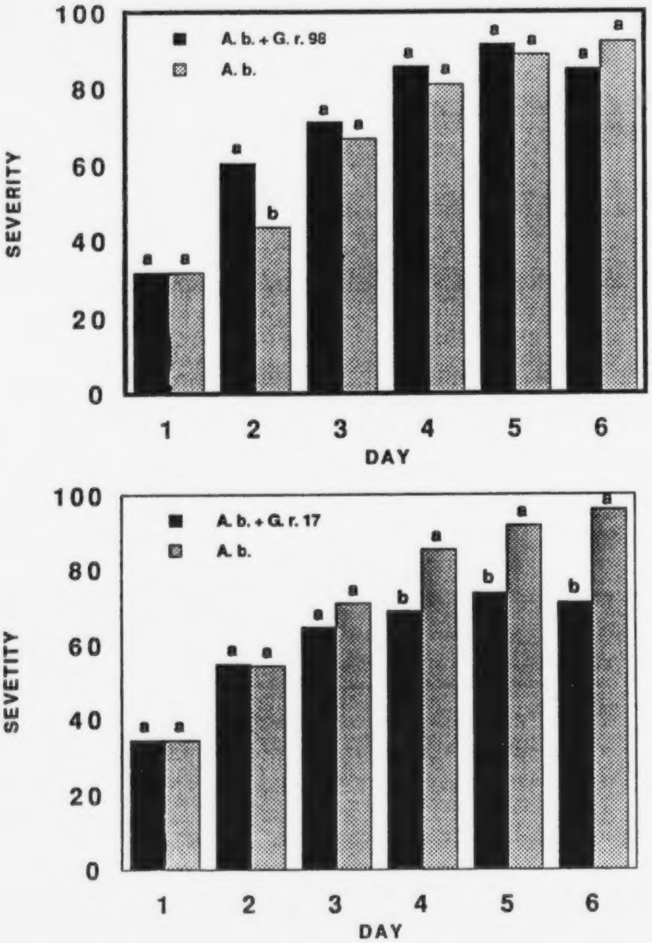


Figure 4.31. Severity of lesions on leaves of Legend caused by *Alternaria brassicae* alone and when conidia of *Gliocladium roseum* isolate 98 (top) and *G. roseum* isolate 17 (bottom) were applied ten hours prior to *A. brassicae.* (A. b. = *Alternaria brassicae*, G. r. = *Gliocladium roseum*, 17 and 98 refer to the isolates of *G. roseum*).

Figure 4.32. Lesion severity 5 days post-inoculation on leaves of Legend when *Alternaria brassicae* was combined with *Gliocladium roseum* isolate 98 (top leaf) and *G. roseum* isolate 17 (bottom leaf). *Gliocladium roseum* was applied prior to *A. brassicae.* The order of droplets for the top leaf from tip to base was *A. brassicae* + *G. roseum* isolate 98, *G. roseum* isolate 98, *A. brassicae* and sterile distilled water. The order of droplets for the bottom leaf from tip to base was *A. brassicae* + *G. roseum* isolate 17, *G. roseum* isolate 17, *A. brassicae* and sterile distilled water. The droplets were in the same order on each half of each leaf.

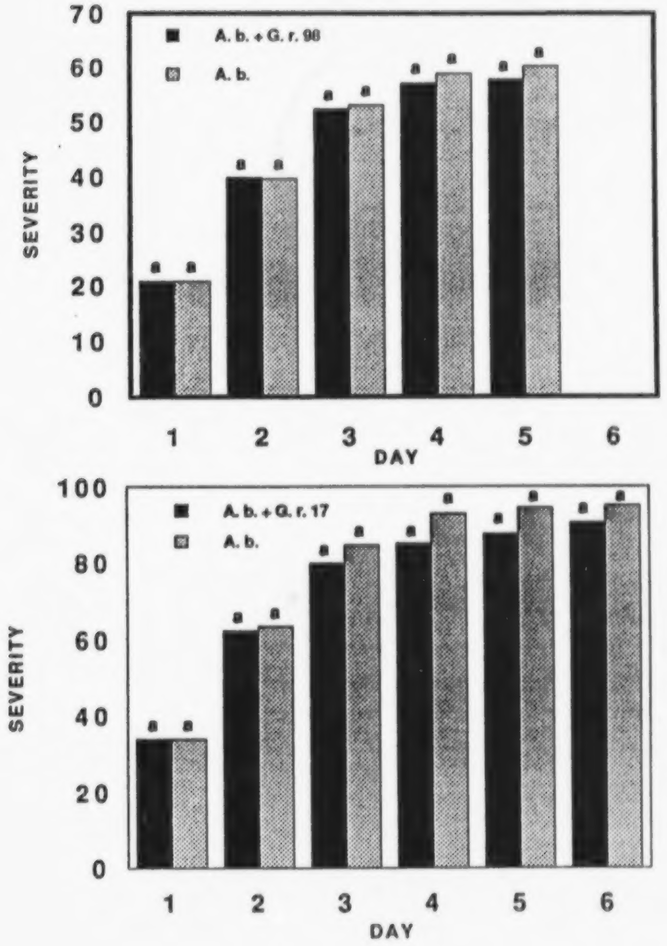
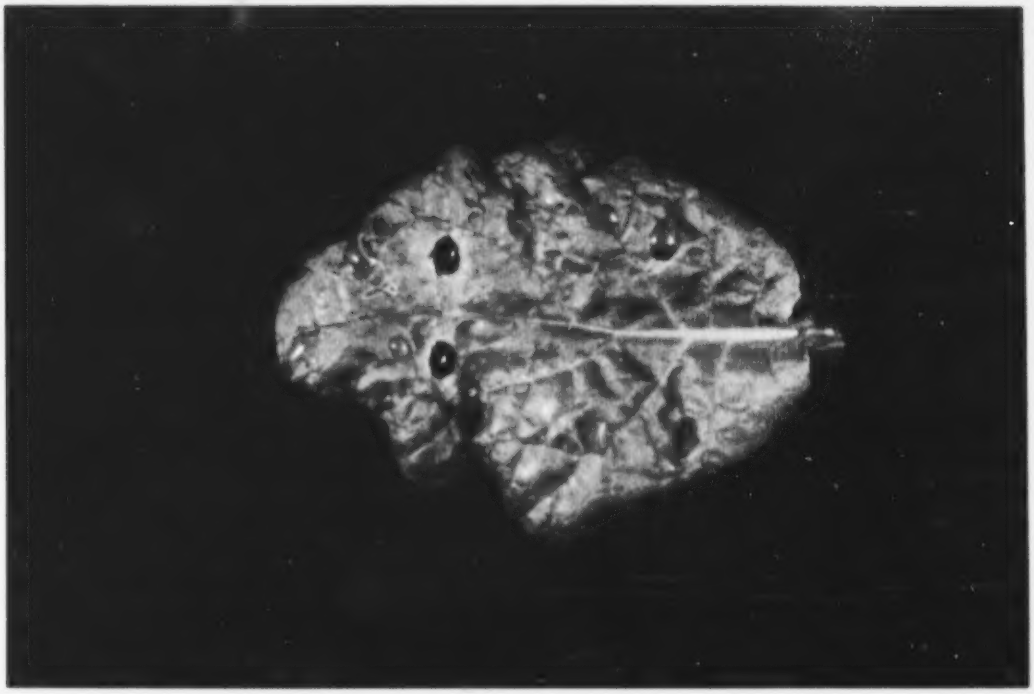


Figure 4.33. Severity of lesions on leaves of Reward caused by *Alternaria brassicae* alone and when conidia of *Gliocladium roseum* isolate 98 (top) and *G. roseum* isolate 17 (bottom) were applied ten hours prior to *A. brassicae.* (A. b. = *Alternaria brassicae*, G. r. = *Gliocladium roseum*, 17 and 98 refer to the isolates of *G. roseum*).

Figure 4.34. Lesion severity 5 days post-inoculation on leaves of Reward when *Alternaria brassicae* was treated with *Gliocladium roseum* isolate 98 (top leaf) and isolate 17 (bottom leaf). *Gliocladium roseum* was applied prior to *A. brassicae.* The order of droplets for the top leaf from tip to base was *G. roseum* isolate 98, *A. brassicae*, sterile distilled water and *A. brassicae + G. roseum* isolate 98. The order of droplets for the bottom leaf from tip to base was *A. brassicae + G. roseum* isolate 17, *G. roseum* isolate 17, *A. brassicae* and sterile distilled water. The droplets were in the same order on each half of each leaf.



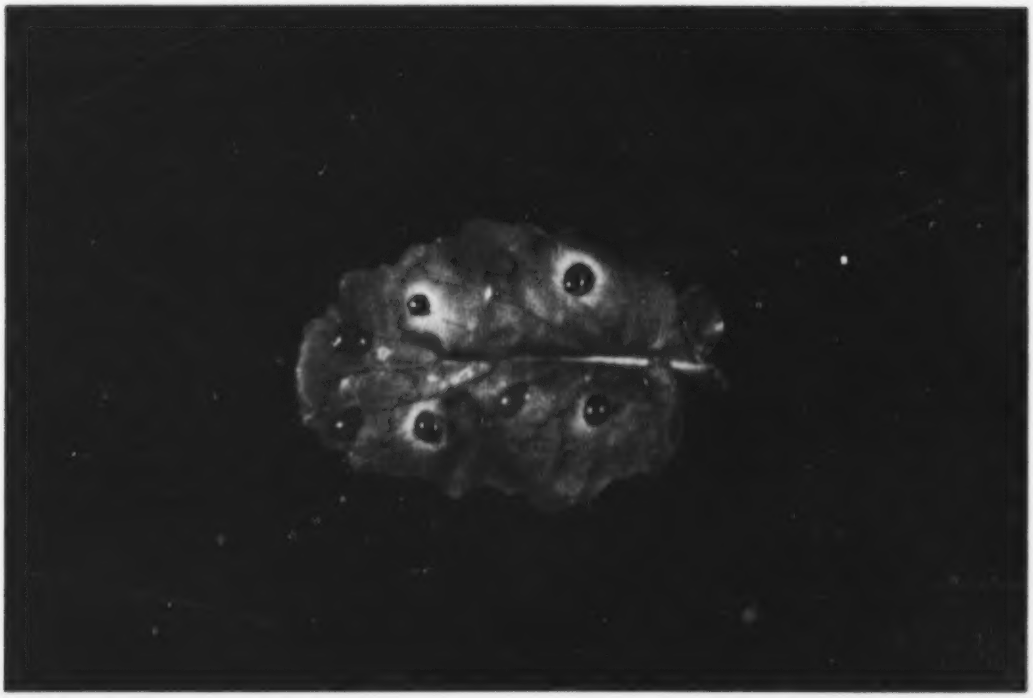


Table 4.4. Days to Appearance of Alternaria Blackspot Symptoms on Excised Leaves of Legend when *Alternaria brassicae* conidia were Applied in combination with the Antagonists or Metabolites of *Myrothecium verrucaria* to Leaves which had Wax Disturbed on One Side

Treatment	*Myrothecium verrucaria* Washed	*Myrothecium verrucaria* Unwashed	*Myrothecium verrucaria* Metabolites	*Gliocladium roseum* Isolate 98	*Gliocladium roseum* Isolate 17
A. brassicae +antagonist/ metabolites	*2.82 a	5.10 a	3.33 a	3.92 a	2.73 a
	†2.57 a	5.00 a	2.25 a	2.50 a	2.08 a
A. brassicae	*3.25 a	2.88 b	3.89 a	2.99 b	3.50 a
	†2.33 a	2.35 b	2.75 a	2.42 a	2.58 a

The range for the standard error of the mean for values presented in this table is from 0.280 to 0.482.

* refers to results from the half leaf with wax undisturbed.

† refers to results from the half leaf with wax disturbed.

Vertical comparisons are indicated with a, b.

Comparisons of days to appearance of disease symptoms were made within wax treatment within antagonist treatment.

Figure 4.35. Comparison of severity of lesions on leaves of Legend (where wax was disturbed on the left half of the leaf) caused by *Alternaria brassicae* alone and when treated with washed conidia of *Myrothecium verrucaria*. (d=disturbed , x=not disturbed, A. b. = *Alternaria brassicae*, M. v. = *Myrothecium verrucaria*, w = washed conidia of *M. verrucaria*).

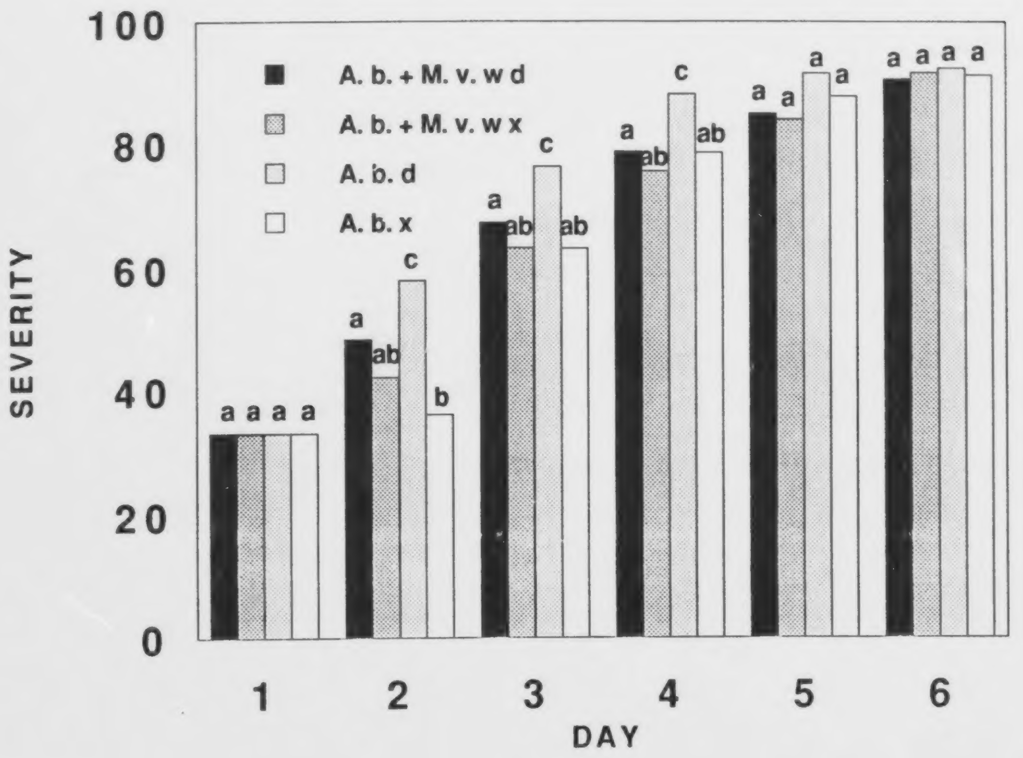

100
80
60
40
20
0
SEVERITY
A. b. + M. v. w d
A. b. + M. v. w x
A. b. d
A. b. x
a a a a
a ab c b
a ab c ab
a ab c ab
a a a a
a a a a
1
2
3
4
5
6
DAY

Figure 4.36. Comparison of severity of lesions on leaves of Legend (where wax was disturbed on the left half of the leaf) caused by *Alternaria brassicae* alone and when combined with *Myrothecium verrucaria* unwashed conidia. (d=disturbed, x=not disturbed, A. b. = *Alternaria brassicae*, M. v. = *Myrothecium verrucaria*, u = unwashed conidia of *M. verrucaria*)

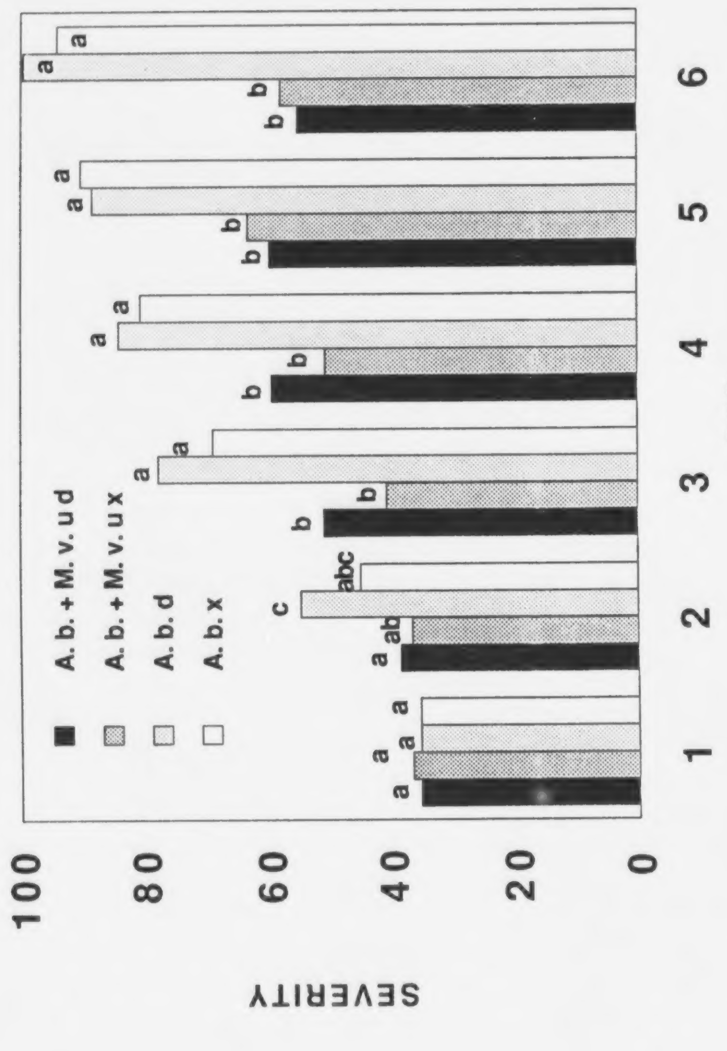
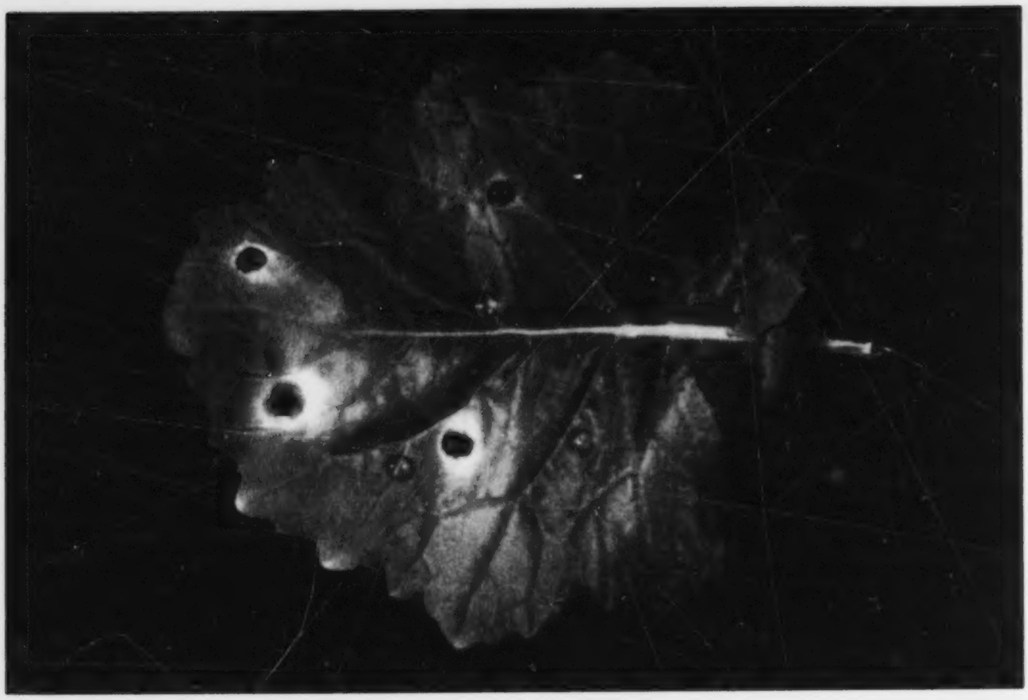

SEVERITY
0
20
40
60
80
100
DAY
1
2
3
4
5
6
A. b. + M. v. u d
A. b. + M. v. u x
A. b. d
A. b. x
a
a
a
a
a
ab
c
abc
b
b
a
a
b
b
a
a
b
b
a
a
b
b
a
a

Figure 4.37. Lesion severity 5 days post inoculation on excised leaves of Legend when *Alternaria brassicae* was combined with washed conidia of *M. verrucaria* (top leaf) and unwashed conidia of *M. verrucaria* (bottom leaf). The fungi were applied simultaneously and wax was disturbed on the left half of each leaf. The order of droplets for the top leaf from the tip to the base was *A. brassicae*, sterile distilled water, *A. brassicae* + washed conidia of *M. verrucaria* and washed conidia of *M. verrucaria*. The order of droplets from the tip to the base for the bottom leaf was unwashed conidia of *M. verrucaria*, *A. brassicae*, sterile distilled water and *A. brassicae* + unwashed conidia of *M. verrucaria*. The droplets were in the same order on each half of each leaf. Note the absence of lesions when *A. brassicae* was combined with unwashed conidia of *M. verrucaria*.



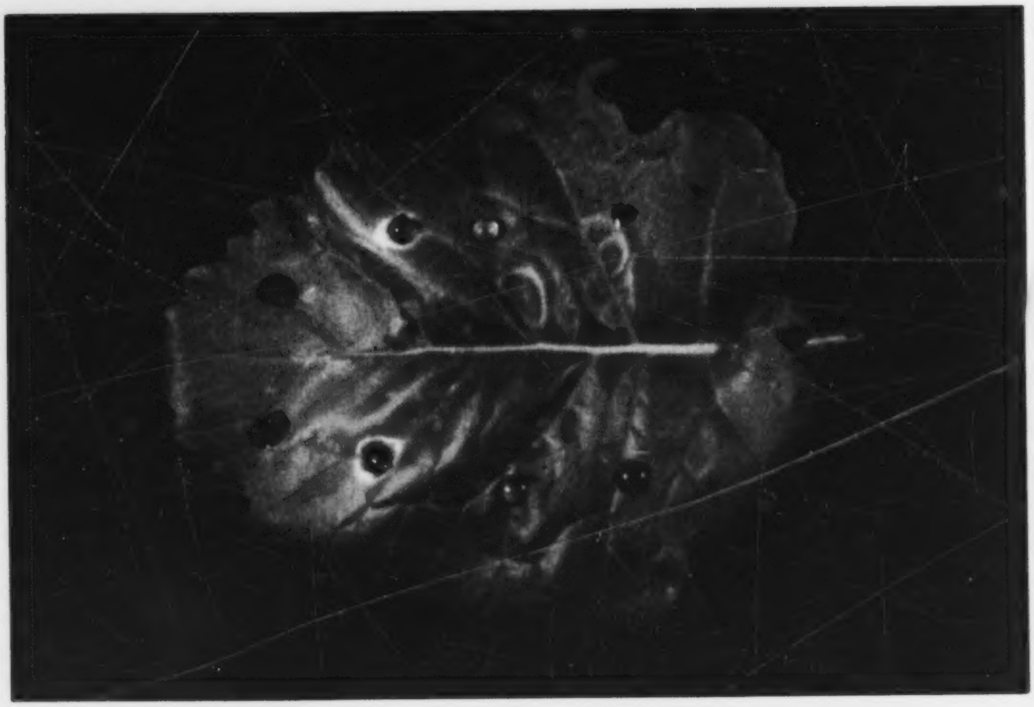


Figure 4.38. Comparison of severity of lesions on leaves of Legend (where wax was disturbed on the left half of the leaf) caused by *Alternaria brassicae* alone and when combined with metabolites of *Myrothecium verrucaria*. (d=disturbed, x=not disturbed, A. b. = *Alternaria brassicae*, M. v. = *Myrothecium verrucaria*, m = metabolites of *M. verrucaria*)

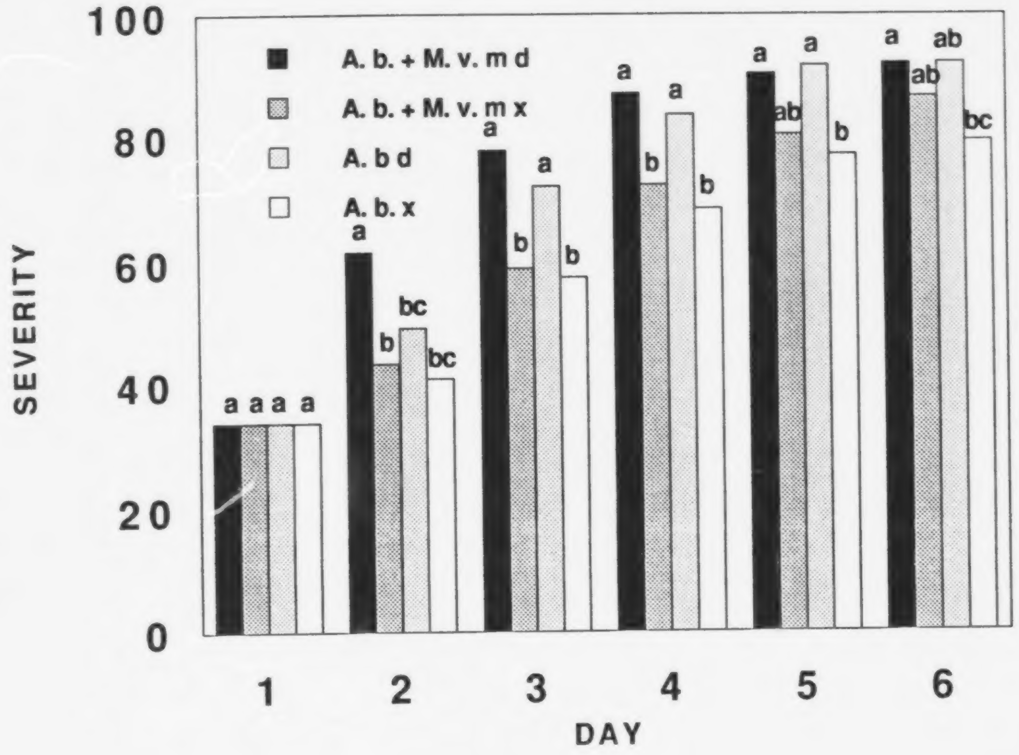

100
80
60
40
20
0
SEVERITY
■ A. b. + M. v. m d
▨ A. b. + M. v. m x
▨ A. b d
☐ A. b. x
a a a a
a b bc bc
a b a b
a b a b
a ab a b
a ab ab bc
1
2
3
4
5
6
DAY

Figure 4.39. Lesion severity 5 days post-inoculation on a leaf of Legend when *Alternaria brassicae* was combined with metabolites of *Myrothecium verrucaria*. The fungi were applied simultaneously and wax was disturbed on the left half of the leaf. The order of droplets from tip to base was *A. brassicae*, sterile distilled water, *A. brassicae* + metabolites of *M. verrucaria* and metabolites of *M. verrucaria*. The droplets were in the same order on each half of the leaf.

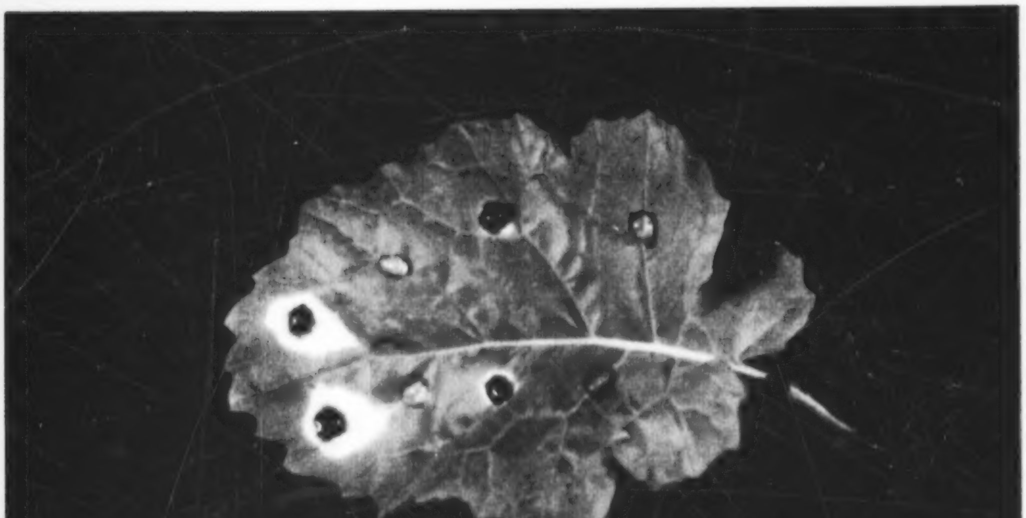


Figure 4.40. Comparison of severity of lesions on leaves of Legend (where wax was disturbed on the left half of the leaf) caused by *Alternaria brassicae* alone and when combined with *Gliocladium roseum* isolate 98. (d=disturbed, x=not disturbed, A. b. = *Alternaria brassicae*, G. r. 98 = *Gliocladium roseum* isolate 98)

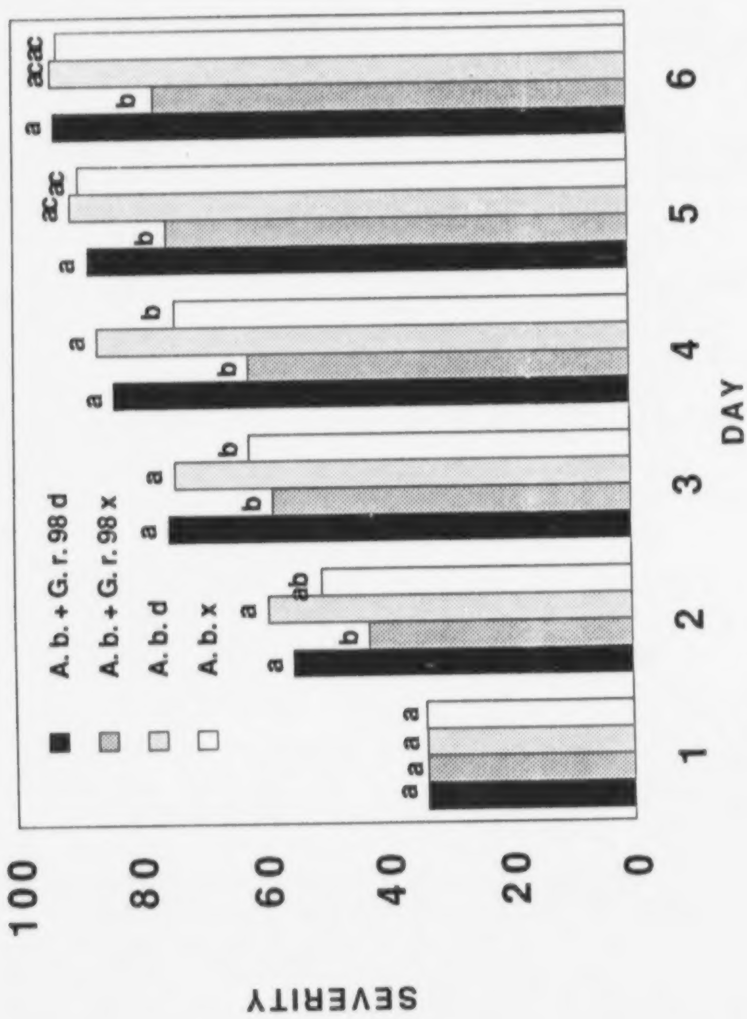

■ A. b. + G. r. 98 d
▨ A. b. + G. r. 98 x
□ A. b. d
□ A. b. x
SEVERITY
100
80
60
40
20
0
DAY
1
2
3
4
5
6
a a a a
a b a ab
a b a b
a b a b
a b ac ac
a b ac ac

Figure 4.41. Comparison of severity of lesions on leaves of Legend (where wax was disturbed on the left half of the leaf) caused by *Alternaria brassicae* alone and when combined with *Gliocladium roseum* isolate 17. (d=disturbed, x=not disturbed, A. b. = *Alternaria brassicae*, G. r. 17 = *Gliocladium roseum* isolate 17)

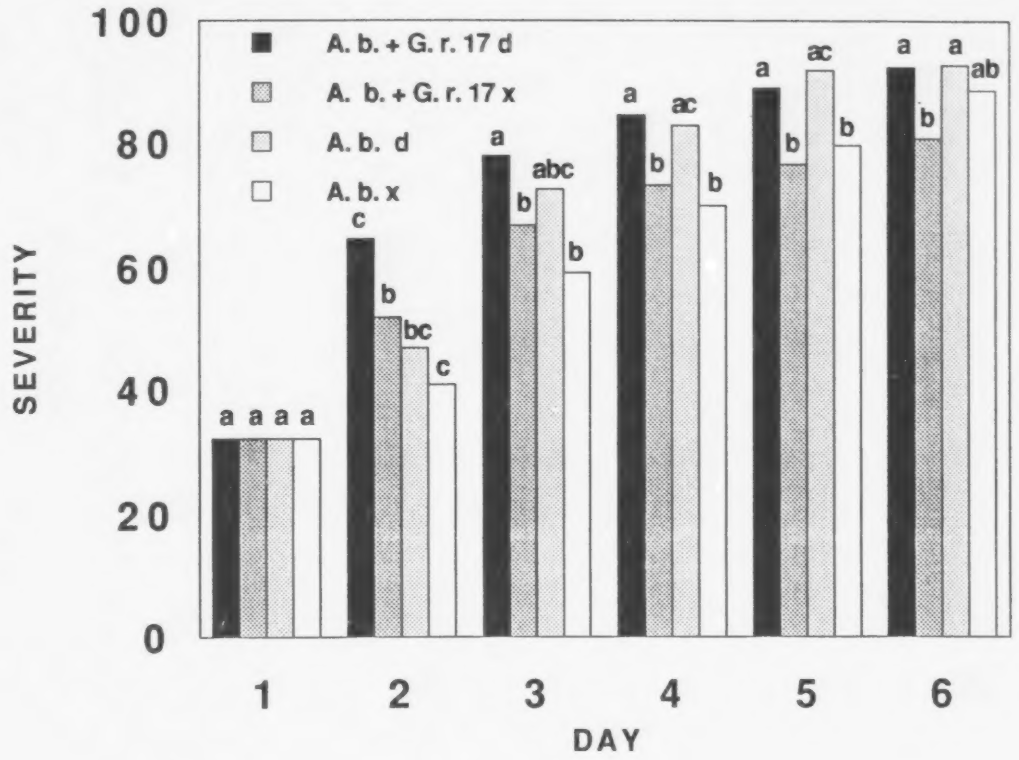

SEVERITY
100
80
60
40
20
0
■ A. b. + G. r. 17 d
▨ A. b. + G. r. 17 x
□ A. b. d
□ A. b. x
a a a a
c b bc c
a b abc b
a b ac b
a b ac b
a b a ab
1
2
3
4
5
6
DAY

Figure 4.42. Lesion severity 5 days post-inoculation on leaves of Legend when *Alternaria brassicae* was combined with *Gliocladium roseum* isolate 98 (top leaf) and *G. roseum* isolate 17 (bottom leaf). Fungi were applied simultaneously and wax was disturbed on left half of the leaf. The order of droplets from the tip to the base for the top leaf was sterile distilled water, *A. brassicae* + *G. roseum* isolate 98, *G. roseum* isolate 98 and *A. brassicae*. The order of droplets from the tip to the base of the bottom leaf was *A. brassicae*, sterile distilled water, *A. brassicae* + *G. roseum* isolate 17 and *G. roseum* isolate 17. The droplets were in the same order for each half of each leaf.

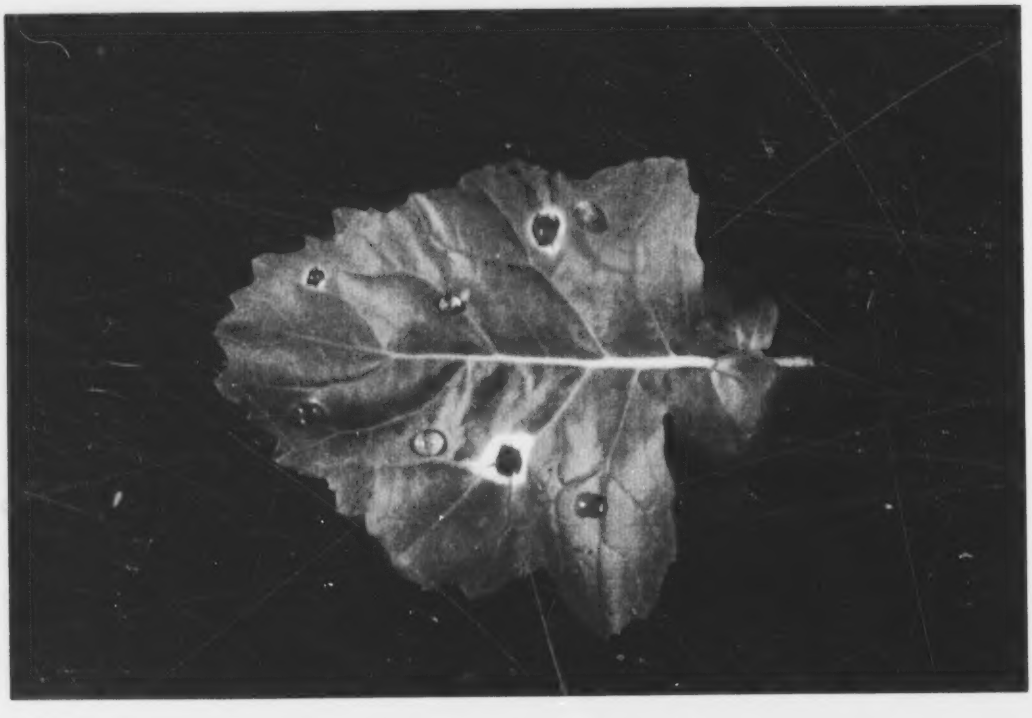


Table 4.5. Days to Appearance of Alternaria Blackspot Symptoms on *In situ* Leaves of Legend and Reward when *Alternaria brassicae* conidia were Applied in combination with the Antagonists or Metabolites of *Myrothecium verrucaria*

Treatment	*Myrothecium verrucaria* Washed	*Myrothecium verrucaria* Unwashed	*Myrothecium verrucaria* Metabolites	*Gliocladium roseum* Isolate 98	*Gliocladium roseum* Isolate 17
A. brassicae +antagonist /metabolites	* 1.55 a	---	1.33 a	1.56 a	1.33 a
	†2.35 a	---	2.11 a	2.22 a	2.00 a
A. brassicae	*1.44 a	1.33 b	1.33 a	1.56 a	1.33 a
	†1.98 a	2.11 a	2.00 a	2.11 a	2.00 a

The range for the standard error of the mean for values presented in this table is from 0.092 to 0.739.

* refers to results for Legend.

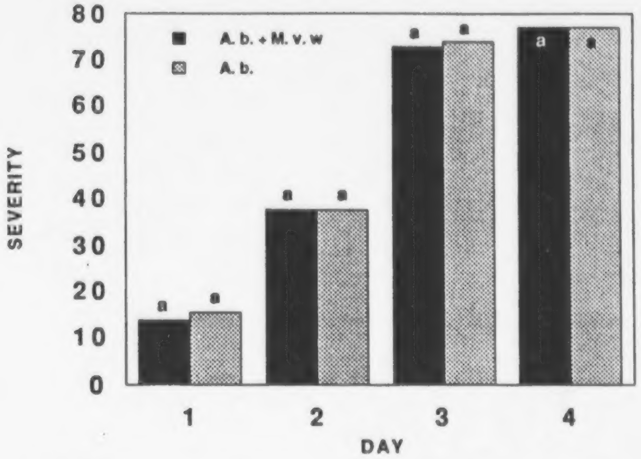
† refers to results for Reward.

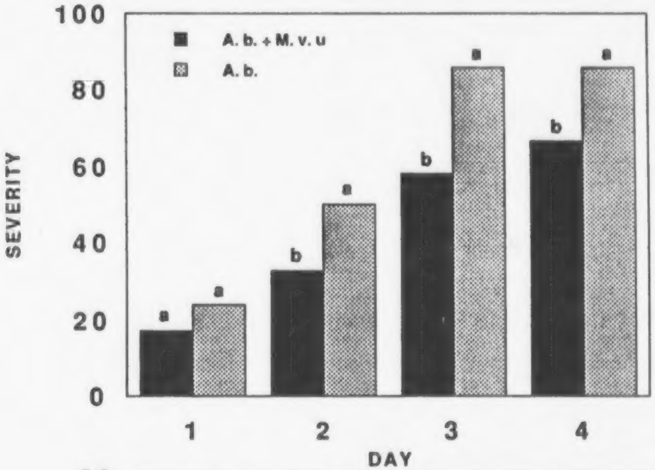
Vertical comparisons are indicated with a, b.

Comparisons of days to appearance of disease symptoms were made within cultivar within antagonist treatment.

Results for Alternaria Blackspot development which were skewed by the presence of disease caused by *M. verrucaria* are not presented in this table.

Figure 4.43. Severity of lesions on *in situ* leaves of Legend caused by *Alternaria brassicae* alone and when combined with washed conidia of *Myrothecium verrucaria* (top), unwashed conidia of *M. verrucaria* (middle) and metabolites of *M. verrucaria* (bottom). (A. b. = *Alternaria brassicae*, M. v. = *Myrothecium verrucaria*, w = washed conidia of *M. verrucaria*, u = unwashed conidia of *M. verrucaria*, m = metabolites of *M. verrucaria*).


■ A. b. + M. v. w
▨ A. b.
SEVERITY
80
70
60
50
40
30
20
10
0
a
a
a
a
a
a
a
a
1
2
3
4
DAY


■ A. b. + M. v. u
▨ A. b.
SEVERITY
100
80
60
40
20
0
a
a
b
a
b
a
b
a
1
2
3
4
DAY

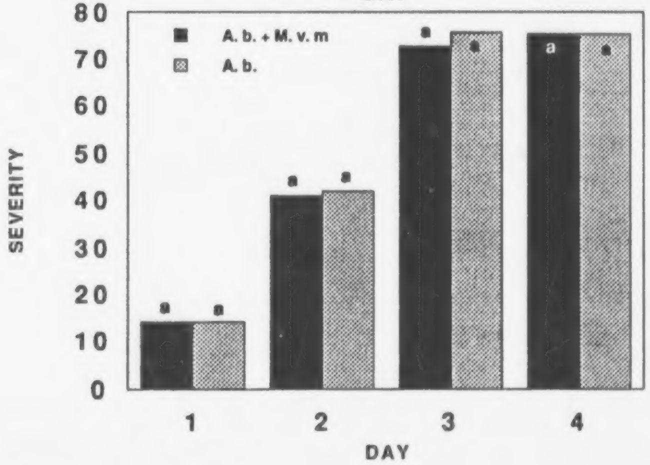

■ A. b. + M. v. m
▨ A. b.
SEVERITY
80
70
60
50
40
30
20
10
0
a
a
a
a
a
a
a
a
1
2
3
4
DAY

Figure 4.44. Lesion severity 4 days post-inocluation on leaves of Legend when *Alternaria brassicae* and antagonists were applied simultaneously. When observed from the base of the leaf, the left side of the leaf was treated with *A. brassicae* in combination with unwashed conidia of *Myrothecium verrucaria* (upper right), washed conidia of *M. verrucaria* (upper left) and metabolites of *M. verrucaria* (bottom). The right side of the leaf was treated with *A. brassicae* only.

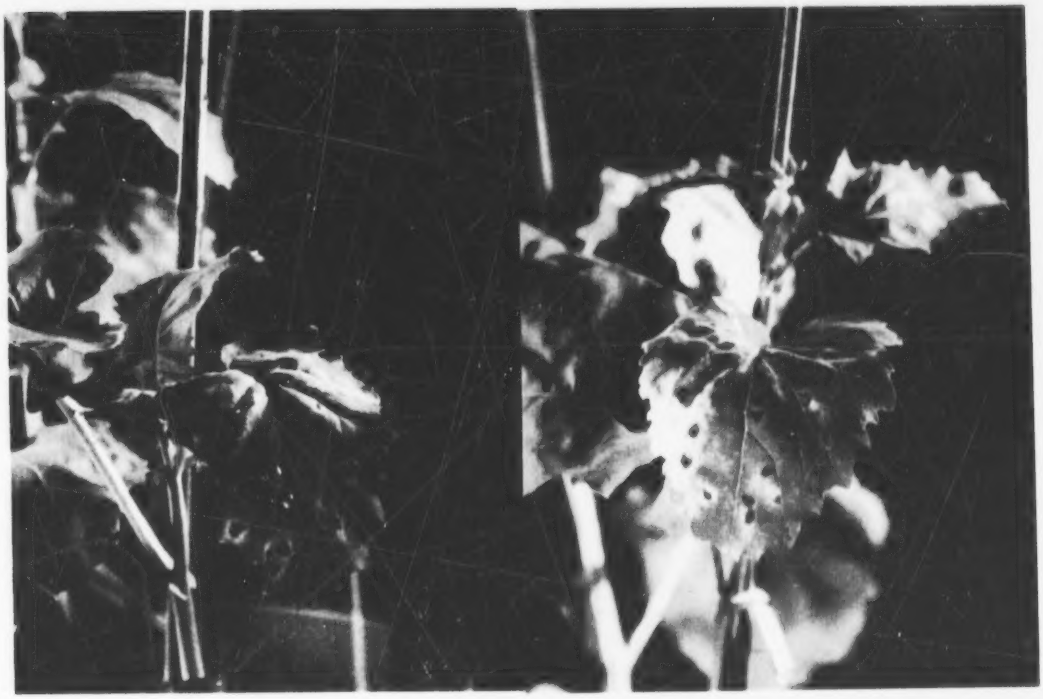


Figure 4.45. Severity of lesions on *in situ* leaves of Reward caused by *Alternaria brassicae* alone and when combined with washed conidia of *Myrothecium verrucaria* (top), unwashed conidia of *M. verrucaria* (middle) and metabolites of *M. verrucaria* (bottom). Note that there was no reduction in lesion severity when *A. brassicae* was combined with unwashed conidia of *M. verrucaria*. This was a result of disease caused by *M. verrucaria*. (A. b. = *Alternaria brassicae*, M. v. + *Myrothecium verrucaria*, w = washed conidia of *M. verrucaria*, u = unwashed conidia of *M. verrucaria*, m = metabolites of *M. verrucaria*).

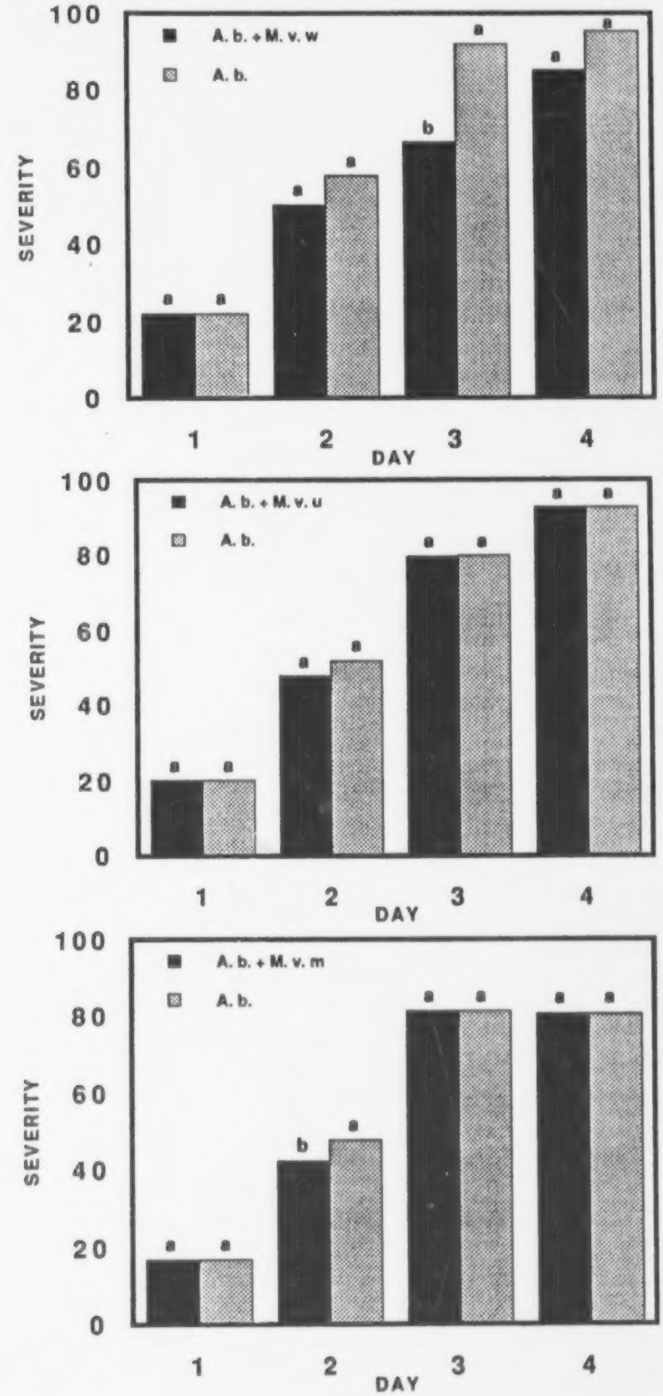
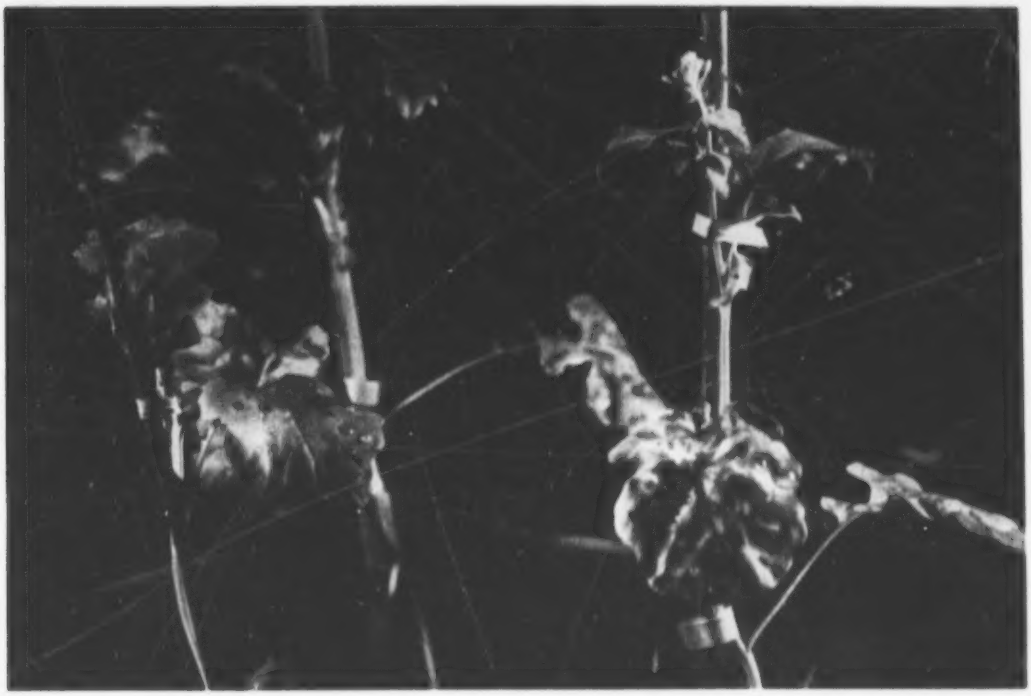

100
80
60
40
20
0
SEVERITY
■ A. b. + M. v. w
▨ A. b.
a
a
a
a
b
a
a
a
1
2
3
4
DAY
100
80
60
40
20
0
SEVERITY
■ A. b. + M. v. u
▨ A. b.
a
a
a
a
a
a
a
a
1
2
3
4
DAY
100
80
60
40
20
0
SEVERITY
■ A. b. + M. v. m
▨ A. b.
a
a
b
a
a
a
a
a
1
2
3
4
DAY

Figure 4.46. Lesion severity 3 days post-inoculation on leaves of Reward when *Alternaria brassicae* and antagonists or metabolites of *Myrothecium verrucaria* were applied simultaneously. When observed from the base of the leaf, the left side of the leaf was treated with *A. brassicae* in combination with unwashed conidia of *M. verrucaria* (top), washed conidia of *M. verrucaria* (lower left) and metabolites of *M. verrucaria* (lower right). The right side of the leaf was treated with *A. brassicae* alone.



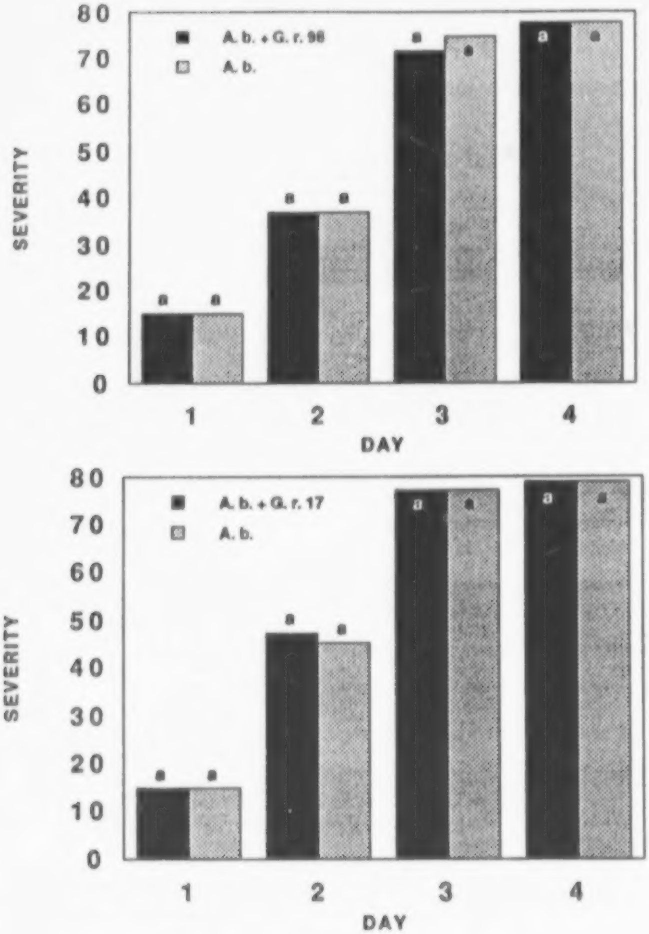


Figure 4.47. Severity of lesions on *in situ* leaves of Legend caused by *Alternaria brassicae* alone and when treated with *Gliocladium roseum* isolate 98 (top) and *G. roseum* isolate 17 (bottom).

Figure 4.48. Lesion severity 4 days post-inoculation on *in situ* leaves of Legend when *Alternaria brassicae* conidia and antagonists were applied simultaneously. When observed from the base of the leaf, the left side of the leaf was treated with *A. brassicae* in combination with *Gliocladium roseum* isolate 98 (left) and *G. roseum* isolate 17 (right). The right side of the leaf was treated with *A. brassicae* only.

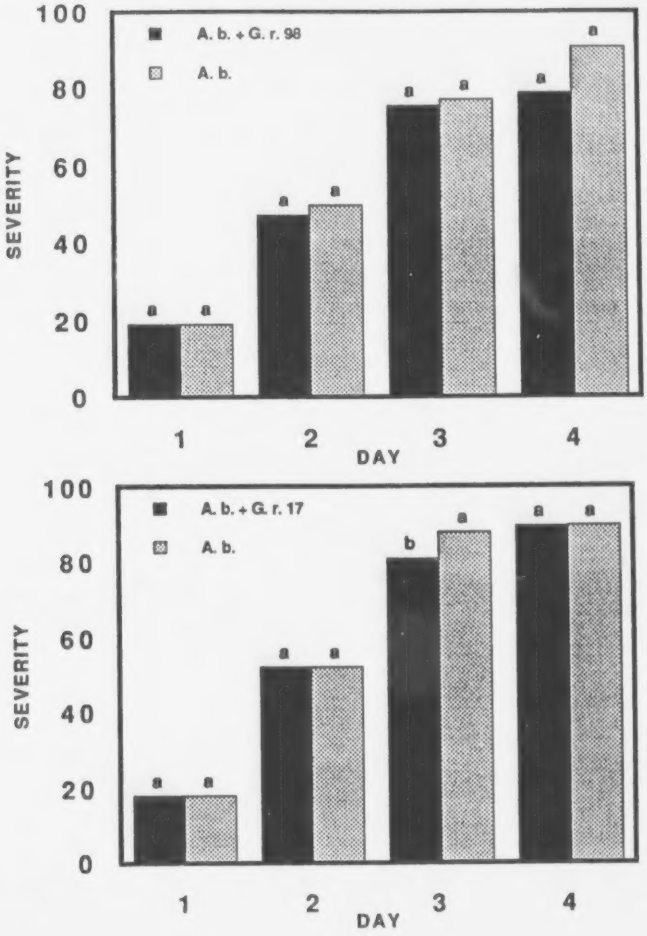


Figure 4.49. Severity of lesions on *in situ* leaves of Reward caused by *Alternaria brassicae* alone and when treated with *Gliocladium roseum* isolate 98 (top) and *G. roseum* isolate 17 (bottom).

Figure 4.50. Lesion severity 3 days post-inoculation on *in situ* leaves of Reward when *Alternaria brassicae* conidia and antagonists were applied simultaneously. When observed from the base of the leaf, the left side of the leaf was treated with *A. brassicae* in combination with *Gliocladium roseum* isolate 98 (top leaf) and *G. roseum* isolate 17 (bottom leaf). The right side of the leaf was treated with *A. brassicae* only.

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CHAPTER FIVE: GENERAL DISCUSSION AND CONCLUSIONS

The goals of this research were to obtain an understanding of the interactions of *Gliocladium roseum* Bainier and *Myrothecium verrucaria* (Alb. & Schw.) Ditmar with *Alternaria brassicae* (Berk.) Sacc. on artificial media and to determine if *G. roseum* and *M. verrucaria* had potential for use as biological control agents of *A. brassicae* on canola. The knowledge gained from the studies conducted could be useful in developing an economically viable system of biological control for Alternaria blackspot.

The effects of the metabolites of *M. verrucaria* on germination of *A. brassicae* conidia and mycelial growth of *A. brassicae* were evident in all studies conducted. These effects were most evident when *A. brassicae* conidia were combined with unwashed conidia of *M. verrucaria*. This is because the greatest concentration of metabolites was carried on the unwashed conidia of *M. verrucaria* in comparison to the washed conidia of *M. verrucaria* and the concentration of metabolites in the crude metabolite preparation. The inhibition of germination and growth of *A. brassicae* was directly related to the concentration of metabolites present, thus it may be possible to control *A. brassicae* to a certain extent through careful use of these metabolites. As *M. verrucaria* produces a number of metabolites belonging to the trichothecenes and no separation or purification work was carried out, it is not known which of these metabolites had the greatest affect on *A. brassicae*. *Myrothecium verrucaria* produces verrucarol and verrucarins A, B and J as well as muconomycins A and B which are scirpene metabolites and anitbiotics with inflammatory properties (Moreau, 1979). Fungistatic activity towards various pathogens has been observed from compounds produced by *Myrothecium* (Gees and Coffey, 1989). The trichothecenes vary in toxicity (Beremand and McCormick, 1992) with the most toxic compounds being inhibitors of the initiation of protein synthesis (Desjardins, 1992). The least toxic of the trichothecenes are those which inhibit the elongation termination of protein synthesis (Desjardins, 1992).

The antagonistic abilities of *G. roseum* and *M. verrucaria* varied on media of differing nutritional value. These differences were seen in both the colony interaction and parasitism studies. The least amount of antagonistic behavior was seen on the media of low nutrient value. This corresponds with the lack of hyphal antagonistic behavior on leaves of Legend and Reward. As the leaf surface is a low nutrient environment, it is not surprising that no antagonism of *A. brassicae* via coiling of the hyphae of *M. verrucaria* or *G. roseum* occurred.

Myrothecium verrucaria is a well documented soil organism with a metropolitan distribution, which is favored by soils high in organic matter (Gees and Coffey, 1989; Papavizas, 1985; Barron, 1983; Purchase, 1974). *Myrothecium verrucaria* has been reported to effectively control diseases caused by necrotrophic parasites, such as *Botrytis cinerea* Pers.:Fr. and *Sclerotinia sclerotiorum* (Lib.) de Bary in controlled environments but generally not in the field (Inglis and Boland, 1992). *Rhizoctonia solani* Kuhn induced damping off has been suppressed by *M. verrucaria* (Cook and Baker, 1983; Ferguson, 1958).

Keinath et al. (1991) successfully used three isolates of *G. roseum* to reduce the viability of microsclerotia of *Verticillium dahliae* Kleb. However, this success may be due to the food base applied with *G. roseum* and the fact that control was achieved in a soil system whereas the present studies were carried out on the leaf surface and no food base was supplied. The success of *G. roseum* in a soil system is not surprising because *G. roseum* is a common soil fungus (Barron, 1983).

Gliocladium roseum has long been known to be an effective mycoparasite, making it a prime candidate for biological control studies. Members of the genus *Gliocladium* have the ability to act as mycoparasites of hyphae and resting structures of plant pathogens in natural soil and *in vitro* (Papavizas, 1985). Examples of its ability to control pathogens, especially melanized resting structures of soilborne phytopathogenic fungi were discussed by Keinath et al. (1991). Three different isolates of *G. roseum* reduced the viability of *V. dahliae* microsclerotia by as much as 100% in soils which were not sterilized. *Gliocladium roseum* has been found in soils in Texas where it was isolated from sclerotia of *Phymatotrichum omnivorum* Duggar and has been observed frequently in Britain on the sclerotia of *Botrytis aclada* Fresen. (syn. *Botrytis allii*). In Canada, there is a negatively correlated relationship between the isolation of *G. roseum* from strawberry plants and the recovery of *V. dahliae* from the same plants. In Swiss cucumber greenhouses *G. roseum* has been used as a biocontrol agent against *Phomopsis sclerotioides* (Sacc.) Sacc. *Gliocladium roseum* has been reported to parasitize *Ceratocystis fimbriata* Ellis & Halst., *Helminthosporium sativum* Pammel, C. M. King & Bakke, *Trichothecium roseum* (Pers.:Fr.) Link, and *Thamnidium elegans* Link: Fr. in culture (Cook and Baker, 1983; Barnett and Lilly, 1962) and *Botrytis allii* Munn (Cook and Baker, 1983; Walker and Maude, 1975).

Gliocladium roseum acts as an antagonist in three ways; (i) as a mycoparasite (e.g. mycoparasitic towards *P. omnivorum*), (ii) through the production of antibiotics (e.g. *in vitro* production of antibiotics against *B. aclada*) and (iii) through competition for infection sites (e.g. competing with *V. dahliae* for infection sites in strawberry crowns) (Keinath et al., 1991). When species of *Gliocladium* parasitize hyphae of other fungi (*in vitro*) morphological changes such as coiling, formation of haustoria, disorganization of host cell contents and penetration of the host occur (Papavizas, 1985).

Gliocladium roseum grown on solid substrates has been demonstrated to successfully control black root rot of cucumber and damping-off of cotton caused by *Phomopsis sclerotioides* Kesteren and *Pythium ultimum* Trow and *Rhizoctonia solani* respectively (Papavizas, 1985).

In these studies, with the exception of the unwashed conidia of *M. verrucaria*, none of the antagonists showed any potential for biological control of *A. brassicae*. In the case of *G. roseum,* lack of control of *A. brassicae* may have been the result of a spore concentration of *G. roseum* which was too low to be effective. Another possibility is that *G. roseum* may need to be applied in conjunction with a food source. However, Papavizas (1985) reported that when *Gliocladium* was applied to soil as conidia with or without a food base, failure of colonization resulted. The same may be true of *M. verrucaria.* Had a food source been supplied, the results of the experiments on leaves may have been different.

Myrothecium verrucaria caused disease on leaves of Legend and Reward. This reduced the possibility of *M. verrucaria* ever being used as a biocontrol agent via conidial application. However, the control of *A. brassicae* by *M. verrucaria* seen on the leaves was not due to hyphal antagonism of *A. brassicae* but was attributed solely to the metabolites of *M. verrucaria* which were carried on the conidia. This indicates that these metabolites may be used in biocontrol studies. A major drawback to the use of these metabolites is that they are toxic to animals, humans, plants, insects, etc. and therefore may be limited in their use.

Although the results obtained through fluorescence microscopy were inconclusive, there is great potential for the use of fluorescence microscopy in observing viability of fungi during mycoparasitism, as was demonstrated by

Barak and Chet (1986). Details of mycoparasitic relationships, such as whether or not the host is alive when attacked have been determined through fluorescence microscopy (Barak and Chet, 1986). Thus, once a reliable protocol has been developed, fluorescence microscopy is an invaluable tool in studies of mycoparasitism. Possible explanations for the inconsistent results obtained from the fluorescence microscopy studies carried out are that the concentrations of dyes and the molarity of the buffer used may not have been optimal for all of the fungi observed. As well, there may have been some inaccuracy in the application of dyes, resulting in concentrations which may have been higher or lower than expected. This may have caused some variability in the results. It may be possible that the cellophane strips used had an affect on the dyes or the uv light, thus affecting the outcome of the studies.

Once the hyphae of *Gliocladium roseum* have grown around the mycelia and spores of susceptible fungi, the host is killed by enzymatic or toxic action, which may be followed by penetration of the victim (Cook and Baker, 1983). Although light microscopy was useful for observing the basic relationships of *M. verrucaria* and *G. roseum* coiling around *A. brassicae* conidia and for the observation of hyphae of these antagonists within *A. brassicae*, the use of scanning and transmission electron microscopes was necessary to determine the intimate details of the relationships. The use of these microscopes in conjunction with sectioning, staining and freeze-fracturing techniques confirmed that *M. verrucaria* and *G. roseum* are mycoparasites of *A. brassicae*. The complete or partial degradation of the cell wall of *A. brassicae* by *G. roseum* and *M. verrucaria*, respectively, indicate that enzymatic or enzymatic and mechanical means were used to penetrate the conidia of *A. brassicae*. *Gliocladium roseum* has been reported to produce both chitinolytic and *B*-1,3-glucocanolytic compounds (Di Pietro et al., 1993; Pachenari and Dix, 1980).

The strength of this work lies in the demonstration that *M. verrucaria* and *G. roseum* are mycoparasites of *A. brassicae*. Turhan (1993) reported that *G. roseum* and *M. verrucaria* are mycoparasitic on *A. alternata*.

Conclusions

1) Metabolites of *M. verrucaria* were responsible for inhibition of germination of *A. brassicae* conidia and growth of *A. brassicae*.

2) *Alternaria brassicae* was effectively controlled with unwashed conidia of *M. verrucaria*. This control was probably due mainly to the metabolites

carried on the conidia. Even though the conidia produce metabolites, it is unlikely that the amount produced over the studies of a shorter time frame (8 hours) would result in any significant inhibition of *A. brassicae*.

3) Washed conidia and metabolites of *M. verrucaria* did not control *A. brassicae* effectively enough to delay disease appearance or reduce disease severity.

4) *Gliocladium roseum* as studied was not effective as a biocontrol agent.

5) *Gliocladium roseum* and *M. verrucaria* were mycoparasitic on A. *brassicae.*

Future Research

Gliocladium roseum overgrew and prevented sporulation of *A. brassicae* on senesced leaves of canola. Therefore, research with *G. roseum* as a fall or early spring application to crop debris should be seriously considered. This type of application may reduce inoculum production by *A. brassicae* thereby reducing incidence and severity of disease in future crops. Keinath et al. (1991) suggested that *G. roseum* may reduce disease caused by *V. dahliae* through the reduction of viability of microsclerotia of *V. dahliae* and therefore suggested the use of *G. roseum* on potato residues in an effort to reduce the formation of microsclerotia of *V. dahliae*.

If a strain of *M. verrucaria* which was not pathogenic to canola could be developed there would also be potential for the use of *M. verrucaria* as an application which would reduce formation of the conidia of the *A. brassicae*. Mostafa (1993) reported that *M. verrucaria* reduced the formation of conidia of *Drechslera teres* (Sacc.) Shoemaker on barley straw.

The metabolites of *M. verrucaria* used in this research were not isolated or purified, but were a crude mixture of several metabolites produced by *M. verrucaria*. It would be interesting to isolate and purify these metabolites and determine which of them had the greatest effect upon *A. brassicae*. This could then be applied as a seed treatment to control infection of seedlings due to seed borne inoculum. It would be of great value if the metabolites produced by *M. verrucaria* could be used as a foliar application, but due to the toxic nature of these compounds, their use may be restricted.

Seed treatment began with fungicides such as the mercurial compounds, progressed to other fungicides such as benomyl and captan which are still in use. Biological seed treatments began with the treatment of legume seeds with

Rhizobium. Control of seedborne diseases via coating seed with bacteria is well established. Control of seedling blight of maize in the field by treating seed with *Bacillus subtilis* is similar to that of seed treated with captan (Rodriguez-Kabana et al., 1977). A seed treatment of *Gliocladium virens* J. H. Miller, J. E. Giddens, & A. A. Foster on cotton seed has been shown to protect the seedlings from damping-off caused by *Pythium ultimum* and *Rhizoctonia solani. In vitro* studies have demonstrated the ability of *G. virens* to parasitize hyphae of *R. solani* while soil studies with *G. virens* show its ability to reduce the number of sclerotia of *R. solani* (Lewis et al., 1989). These results indicate that there may be a possible place for *G. roseum* as a seed treatment to prevent infection of seedlings from seed-borne *A. brassicae.* Studies have been conducted on the seed-borne phase of *A. brassicicola* (Schwein.) Wiltshire which indicate that there is potential for the use of antagonists in controlling seed-borne disease caused by various *Alternaria* species (Wu and Lu, 1984).

Another suggestion for future research is to study any internal changes, such as vacuolation or disorganization of cell contents, which may occur when *A. brassicae* is attacked by *G. roseum* or *M. verrucaria.* Also, a study of the enzymes produced at the point of penetration may prove interesting.

On the genetic level, it may be interesting to study the genes of *M. verrucaria* which control the formation of metabolites. If the production of metabolites could be enhanced, the possible use of these metabolites as a type of biological control would be increased.

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